Supplement to the

"Proceedings of the Indian Academy of Sciences"

PROCEEDINGS

OF THE

INDIAN ACADEMY OF SCIENCES

VOL. XXXIX

SECTION B

BANGALORE CITY
PRINTED AT THE BANGALORE PRESS, MYSORE ROAD
1954

Q
73
.I36
Sect. B
v.39

CONTENTS

SECTION B—VOL. XXXIX

PAGE

No. 1—January 1954

Effect of Maleic Hydrazide on Growth, Bolting Behaviour and Keeping Quality of Indian Radish (*Raphanus sativus* L.) . . . . . R. S. Choudhri and V. B. Bhatnagar 1

Studies on the Red Halophilic Bacteria from Salted Fish and Salt . . . . . R. Venkataraman and A. Sreenivasan 17

On Some Metamorphosing Stages of Eels (Murænidæ) from the Estuary of the Burhabulong River, Orissa State . V. R. Pantulu and S. Jones 24

No. 2—February 1954

Studies on Some Aspects of Bioassay of Stomach Poisons . . . . . S. Pradhan and M. G. Jotwani 37

Relation between the Age of Rice Plant and Its Susceptibility to Helminthosporium and Blast Diseases . S. Y. Padmanabhan and D. Ganguly 44

A Contribution to the Embryology of Bombacaceæ . . . C. Venkata Rao 51

On the Life-History and Bionomics of the Carp Minnow, *Chela phulo* (Hamilton) . . . . . K. H. Alikunhi and H. Chaudhuri 76

No. 3—March 1954

Fish Mortality in the Rock Pools of a Quarry near Virudhunagar (Ramanathapuram District), Madras . . P. I. Chacko and R. Srinivasan 91

Digestion of Cellulose by the Mound Building Termite, *Termes* (*Cyclotermes*) *obesus* (Rambur) . . . . . J. N. Misra and V. Ranganathan 100

Contribution to Our Knowledge of the Physiological Anatomy of Some Indian Hydrophytes. I. The Stem of *Cæsulia axillaris* Roxb. . . . . . M. V. Mirashi 114

A New Member of the Euglenineæ, *Protoeuglena noctilucæ* gen. et sp. nov., Occurring in *Noctiluca miliaris* Suriray, Causing Green Discoloration of the Sea Off Calicut . . . . . R. Subrahmanyan 118

A Contribution to the Life-History of *Artocarpus lakoocha* Roxb. . . . . . I. Banerji and A. Hakim 128

PAGE

No. 4—April 1954

Studies on the Black Rot of Coffee. I. The Disease in South India and Some General Considerations . . . . . K. T. Mathew 133

Pectic Changes in Pears During Storage and Ripening . . . . . W. B. Date and Elmer Hansen 171

No. 5—May 1954

Studies on the Black Rot of Coffee. II. Nutritional Requirements of *Pellicularia koleroga* Cooke with Special Reference to Growth Substances . . . . . K. T. Mathew 179

Studies in the Chenopodiaceæ. II. Embryology of *Arthrocnemum indicum* Moq. . . . . . T. S. Mahabale and I. N. Solanky 212

No. 6—June 1954

Effect of Fluorine on the Composition of Bones: Changes in the Composition of Bones of Rats—Part I . . . . . T. K. Wadhwani 223

Availability of Calcium in Some of the Leafy Vegetables . . . . . S. C. Devadatta and T. C. Appanna 236

Rooting Responses in Isolated Tropical Leaves . . . . . B. Samantarai and T. Kabi 243

A Contribution to the Embryology of *Olax wightiana* Wall. . . . S. Shamanna 249

EFFECT OF MALEIC HYDRAZIDE* ON GROWTH, BOLTING BEHAVIOUR AND KEEPING QUALITY OF INDIAN RADISH (*Raphanus sativus* L.)

By R. S. Choudhri, M.Sc., Ph.D. (Cornell, U.S.A.), D.Sc.
(Professor of Agricultural Botany and Physiologist, College of Agriculture, Banaras Hindu University)
AND
V. B. Bhatnagar, M.Sc. (Ag.)
(Research Scholar)

Received June 3, 1953
(Communicated by Prof. P. Parija, F.A.Sc.)

Introduction

Indian radishes weigh each from a ¼ lb. to as much as 10 lb. or even more, depending upon the variety. The roots are ready for the market approximately 4-5 weeks after sowing when they are tender and good to eat both raw and cooked. But most varieties are quick flowering, and with the advent of this stage, the roots generally become hard, woody, often hollow too and lose their edible character. Every grower is, thus, confronted with the problem of quick disposal of his produce with the result that market gets unusually flooded and the crop fetches but small returns. Any means that could inhibit flowering in the crop, partially or wholly, will, for evident reasons, not only be appreciated by the growers but by the consumers also.

The time of flowering in plants is, seemingly, largely dependent upon certain factors of environment (*cf.* Murneek and Whyte *et al.*, 1946, and Whyte, 1946) of which temperature and light have been recognized by far the most important ones (Lundegardh, 1931, and Garner and Allard, 1920). There is enough evidence to indicate that temperature and photoperiods control flowering through effect on some process in leaves, perhaps on the production of a flowering hormone—the 'florigen' (Cailahjan, 1937) or on a certain phytohormone complex (Cholodny, 1939) or on the so-called factors A, B and C (Hamner, 1942).

Hitchcock and Zimmerman (1935), Thimann and Lane (1938), Clark and Kerns (1942), Cooper (1942) and van Overbeek (1945) have reported

* MH—30 Formulation was kindly supplied by the United States Rubber Coy., Naugatuck, Chemical International Division, N.Y., and to whom the authors are greatly thankful.

a hastening in flowering of certain crops through application of synthetic growth substances. Dostal and Hosek (1937), Thurlow and Bonner (1947), Wittwer, Coulter and Carolus (1947) and others have, contrariwise, reported inhibition effects on flowering following application of such substances. Bonner and Bandurski (1952) have critically reviewed the effects of auxin on flowering.

Besides the above, investigations on maleic hydrazide (1, 2-dihydropyridazine-3, 6-dione), commonly designated as MH, have created considerable amount of scientific interest in recent years. White (1950), White *et al.* (1950) and Kennard *et al.* (1951) obtained delayed blossoming in raspberries, Hubbard and Rowland (1952) in spinach, Choudhri and Bhatnagar (1953) in onions and Kosar and Thompson (1951) and Choudhri and Bhatnagar (1953) in lettuce through treatment of plants with maleic hydrazide. But observations are on record where lighter dosages of MH have caused stimulating effects on flowering in lettuce, when applied in early stages of plant growth (Barnard and Warden, 1950; Crafts *et al.*, 1950; and Choudhri and Bhatnagar, 1953).

Our experience that maleic hydrazide could profitably be used as an effective flower suppressent, lead us to extend these investigations on radish crop, where the problem of quick flowering is rather acute. This was considered an important issue towards the successful production of several vegetable crops and more specially the radishes.

MATERIAL AND METHODS

Radish (*Raphanus sativus* L., N.O. *Cruciferæ*) *var.* Newar seeds were grown in the experimental field in rows 1′ apart, fairly closely. After the completion of germination, when seeds had passed into 2-leaf stage, thinning was done in a manner so that plant to plant distance approximated 6″. Watering and hoeing were carried on at regular intervals.

The experimental field was later on divided into 24 plots, each $6' \times 8'$ in size. Six such plots, which on an average had a total of 540 plants were used for one treatment. The selection of plots for one treatment or the other was made at random. Three types of treatments using maleic hydrazide in three different concentrations of 0·05, 0·10 and 0·20 per cent. were taken recourse to. No wetting agent was used with maleic hydrazide. A fourth group of six plots was employed as check.

Spraying of tops with maleic hydrazide was done 6 weeks after planting before flowering had ensued. A knapsac sprayer was used for this purpose and the solution was sprayed at the rate of 40 gallons to an acre.

Observations

Growth Characteristics.—Effects of MH spray were well visible after about a week and the first observation was recorded a fortnight after the treatment. Fresh and dry weights of both tops and roots were recorded at regular intervals (Table I).

A survey of the data presented in Table I shows that both the fresh and dry weights of tops and roots were significantly affected by MH application. The effectiveness, however, differed with the concentration of the solution applied. In the case of roots maximum effectiveness, resulting in increased fresh and dry weights, was shown by 0·10 per cent. maleic hydrazide. Lighter and heavier dosages characterized peculiar effects as at most stages of root growth fresh weights were a little lower than those of the checks but dry weights were higher. This seems to indicate that maleic hydrazide affected the turgidity of roots to a certain extent.

Effects of maleic hydrazide on the tops of the plants were still peculiar. There was invariably a drop in the fresh weights and a rise in the dry. Here again, highest dry weight was recorded following treatment with 0·10 per cent. MH. It seems that despite certain visual effects, apparently injurious to tops, the photosynthetic mechanism was not adversely affected. The action of maleic hydrazide in these cases may rather be taken stimulative, depending upon the suitability of the dose, as dry weights of both tops and roots were on the increase.

As roots in radish plants are really the organs of economic importance, it was felt necessary to compute root/top ratio both on fresh and dry weight basis. These findings have been reported in Table II. It is again clear that this ratio is on the rise following MH applications. The high root/top ratio, seemingly, results from a lowering in the fresh weight of tops. Highest root/top ratio, both when computed on fresh or on dry weight basis, is obtained at a concentration of 0·10 per cent. MH. A trend towards a larger increase in root weights following MH treatment seems to owe to a more rapid and a greater transport of food from tops to roots, perhaps because of suppression of flower formation.

Of further interest it was noted that roots of plants treated with maleic hydrazide were more tender, turgid and less woody at all stages of growth relative to controls (Fig. 1). In most of the control plants, when three months old, roots became useless and practically unedible either due to excessive wood development or to extreme hollowness. On the contrary, over 70 per cent. of the roots from the treated plants remained succulent and juicy at this age. These results indicate that maleic hydrazide under suitable dosage

TABLE I

Average Fresh Weight in gm. (F.W.) and Dry Matter Percentage (D.W.) of Tops and Roots for Radish Crop at Successive Stages of Growth Following Maleic Hydrazide (MH) Application; the Same at the Time of Treatment being

	Root		Top	
	F.W.	D.W.	F.W.	D.W.
	44·3	3·29	247·3	6·98

Treatment	2 weeks after treatment				4 weeks after treatment				6 weeks after treatment			
	F.W. roots	D.W. roots	F.W. tops	D.W. tops	F.W. roots	D.W. roots	F.W. tops	D.W. tops	F.W. roots	D.W. roots	F.W. tops	D.W. tops
Untreated (Control) ..	540·7	4·51	321·0	8·00	589·1	5·01	427·0	9·34	631·7	5·31	482·0	10·76
0·05% MH	535·1	5·90	255·8	10·30	585·3	6·25	301·3	12·05	613·8	6·95	338·1	14·31
0·10% MH	576·3	7·54	223·7	12·70	617·4	8·43	241·4	14·80	661·0	9·05	269·7	17·42
0·20% MH	528·1	6·12	218·0	10·64	591·4	6·80	234·0	11·98	609·9	7·95	247·1	15·03

TABLE II

Relative Root/Top Ratio for Treated and Untreated Radish Plants at Second, Fourth and Sixth Weeks after Treatment with Maleic Hydrazide (MH), Computed on Fresh and Dry Weight Basis

Treatment	Fresh Weight Basis			% Dry Weight Basis		
	2 weeks	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks
Untreated ..	1·684	1·379	1·310	0·563	0·536	0·493
0·05% MH ..	2·091	1·942	1·815	0·572	0·518	0·485
0·10% MH ..	2·576	2·557	2·450	0·593	0·565	0·556
0·20% MH ..	2·422	2·527	2·468	0·575	0·567	0·528

concentration, when applied at opportune time, holds considerable promise for crop and quality improvement.

Bolting Behaviour.—Treatment of plants with maleic hydrazide was carried on when they were 1½ months old. By this time, none of the plants had bolted. After a lapse of another 2 weeks, however, the check plants exhibited bolting to an extent of 22 per cent., which gradually increases reaching a level of 57 per cent. 4 weeks after treatment and 89 per cent. after 6 weeks (Table III).

TABLE III

Percentage Bolting for Treated and Untreated Radish Plants at Second, Fourth and Sixth Weeks after Treatment with Maleic Hydrazide (MH); None of the Experimental Plants Exhibited Bolting at the Time of Treatment

Treatment	2 weeks	4 weeks	6 weeks
Untreated ..	22	57	89
0·05% MH ..	Nil	11	27
0·10% MH ..	Nil	5	13
0·20% MH ..	Nil	4	9

In treated plants bolting was rare and that too greatly delayed. Only a few plants bolted a month after treatment and a few more later (Table III). The intensity of flowering depended upon the concentration of maleic

hydrazide used, higher the concentration, lesser the chances of bolting. Bolting was, thus, markedly suppressed as a result of MH spray.

The suppression effect on the initiation and development of the bolting shoot following MH application was further evident on an examination of the number of scape branches and the size of the scapes formed on few plants (Table IV). A marked reduction on either side was recorded as the concentration of the maleic hydrazide solution increased. The few scapes formed on few plants following 0·20 per cent. MH application were short and but sparsely branched (Fig. 2).

TABLE IV

Certain Morphological Observations Recorded on Maleic Hydrazide Treated Radish Plants Relative to Untreated Controls; All Observations Gathered Ten Weeks after Treatment

MH Concentration %	0·00	0·05	0·10	0·20
Average Height of Scape (cm.) ..	147·5	87·0	61·3	33·7
Average No. of Branches/Scape ..	105·0	35·0	18·0	7·6

Besides delay or suppression in bolting, a pronounced effect on the fruiting behaviour was also noticeable. Fruits formed on the treated plants were too few, only 8 and 3 per cent. respectively on the application of 0·05 and 0·10 per cent. MH. The size of the fruits from treated plants was small and seeds formed were few (Table V). The controls exhibited 100 per cent. fruit set, bearing larger fruits with 5 to 7 seeds (Fig. 3).

TABLE V

Fruiting Responses in Radish Plants Following Treatment of Young Tops with Maleic Hydrazide; All Observations Gathered 10 Weeks after Treatment

MH Concentration (%)	0·00	0·05	0·10	0·20
Plants Setting Fruits (%)	100	8	3	Nil
Average No. of Fruits per Plant ..	721	90	44	Nil
Average Fruit Size (Inches) ..	3·0	1·7	1·2	Nil
No. of Seeds per Fruit	5–7	0·2	0–1	Nil

Miscellaneous Responses.—It has been noticed that there was a set-back in the formation of the flowering shoot following maleic hydrazide sprays. In a few cases, however, more particularly where the concentration of maleic hydrazide was low, scapes did develop and were apparently not abnormal. On the contrary, the few scapes formed on plants treated with 0·20 per cent. MH, showed peculiar 'formative effects' (Fig. 2). They were fasciated and all new leaves formed on these scapes turned strap shaped. The scape internodes exhibited but stunted growth and the number of flower buds formed was exceedingly low, and those too under-developed. Almost all flower buds turned elliptical and flat. Such buds were shortlived and readily abscissed at the point of attachment of the floral whorls to the thalamus. Yellowing of buds, prior to abscission, was a characteristic feature of such treatments.

On dissecting the flower buds obtained from the treated plants, the stamens were observed to be sessile and the anthers flaccid and shrivelled and almost devoid of pollen. This latter observation suggests the possibility of meiosis being affected at one stage or the other. Other abnormalities such as those leading to the reduction of petals from 4 to 3 and lack of development of other floral parts were not uncommon. These features more commonly characterized plants treated with 0·10 or 0·20 per cent. MH.

A noteworthy feature of radishes from treated plants was a marked deviation in their pungency relations. Radishes from treated plants were found to be less pungent relative to controls. They were on the contrary sweeter in taste.

After-Effects Following Treatment of Young Bolted Shoots.—The above responses regarding bolting behaviour in radish related to plants where MH treatments were administered much before the flowering stage. In a few cases, effectiveness of MH spray in a concentration of 0·10 per cent. was studied on plants where bolting had just commenced. It was interesting to note that following treatment further formation and development of flowers practically stopped. Petals of flowers already formed did not expand, the anthers turned flaccid and lacked in pollen. Most buds and flowers changed their colour and subsequently abscissed. Fruit setting, if at all, was greatly impaired and the few fruits that had developed by this time remained small and seedless. A marked reduction in the number of scape leaves and scape branches was also noticed (Fig. 4). The suppression effect on the growth and the development of the bolting shoot was associated with the negation of the apical dominance and acceleration in the development of buds in the axils of the radical leaves (Fig. 4).

Water-Holding Capacity of Marketable Radishes and Its Influence on Their Keeping Quality.—With a view to judge whether there was any effect of the MH spray on the water-holding capacity of radishes, a large number of treated and untreated plants with roots intact were pulled out from the field, only a week after treatment. Tops of such plants were immediately cut off to avoid loss of water through transpiration. Such radishes were stored in the laboratory at room temperature ($21° \pm 1·5°$ C.) along with controls. Wet weight determinations at regular intervals gave an estimate of the relative tendency of treated and untreated radishes to lose water (Table VI).

TABLE VI

Average Wet Weights in Gm. of Radish Roots (Each) from Maleic Hydrazide Treated and Control Plants Pulled One Week after Treatment and Weighed for Water Loss at Weekly Intervals

Treatment	Initial	1 week after	2 weeks after	3 weeks after	% water loss after 3 weeks
Untreated (Control) ..	542	385	246	134	75·27
MH Treated (Conc. −0·10%)	553	465	386	317	42·67

It was interesting to note that radish roots from untreated plants weighing as much as 542 gm. decreased to a total fresh weight of 134 gm. after a 3-weeks period while those from plants treated with 0·10 MH weighed as much as 316 gm. against 553 gm. of the original fresh weight after the lapse of the same period of 3 weeks. Water loss in the radishes from treated plants was thus much less (about 43 per cent.) as compared to controls, where the same was no less than 75 per cent. The treated roots keep better relative to controls, which became pithy, hollow and blackish within, after a few days of storage (Fig. 5). These characters of radishes from the treated plants would certainly add to their keeping-quality and thus appear to bear great economic significance.

It is well known that growers dig out radishes for marketable purposes in bulk and those that are left behind unsold lose their turgidity fairly rapidly. To avoid this, vegetable sellers keep their stock of radishes either under wet cloth or under frequent spray of water. Even with such precautions heavy losses accrue and radishes usually shrink and become unattractive. If MH spraying of radish plants could help the water-holding capacity of radishes, as the observations in this investigation indicate, heavy losses so common

to marketable vegetables could be controlled, atleast, to some extent and the period of availability of fresh vegetables would prolong.

Discussion

Treatment of radish tops with maleic hydrazide solution resulted in an inhibition in shoot growth under all concentrations used in this investigation, the intensity of inhibition depending upon the strength of MH dosage. It is, however, noteworthy that wet weight of roots either remained unaffected or exhibited small increases under suitable concentrations (0·10% MH). The dry matter percentage of tops and roots of treated plants, on the other hand, showed a definite increase over the controls. Root/top ratio, thus calculated on either fresh or dry weight basis was invariably characterized with higher values for treated plants relative to controls. Such increases evidently accrue from non-injurious effects that associate root growth following MH treatment of tops. Considering the root/top ratio, MH dosage at 0·10 per cent. level was more useful than other concentrations of higher or lower order. Currier *et al.* (1951) working with barley plants also obtained dry weight increases following MH applications at 0·20 to 0·40 per cent. These workers explain their findings on the basis of larger accumulations of fructosans and polysaccharides in the treated plants. Roots in radish, which form the most important organs of storage, are likely to have been similarly affected by MH treatments.

It was noted that the roots from untreated control plants gradually turned woody, hard and hollow as their age advanced, in field conditions. Roots from treated plants, contrariwise, remained tender, turgid and succulent for over a period of 1 month and more relative to controls. Burleson and Hubbard (1951) found MH in concentrations of 0·75–1·00 per cent. to be effective enough to control secondary growth in cotton plants and a complete inhibition in this direction was recorded by Hall (1951) in as low a concentration of MH as 0·48 per cent. Non-development of wood in radish roots even in as low a concentration as 0·05 per cent. may be explained alike. It seems different types and species of plants differ markedly in their responsiveness to maleic hydrazide.

The mechanism of action of MH on secondary growth in plants may better be elucidated on a detailed histo-chemical study of treated and untreated plant organs. One thing, of course, is obvious that maleic hydrazide inhibits, apart from the activity of terminal meristematic cells, the growth of intercalary meristems, including those of cambium cells. It also seems likely that lignification and thickening of cell walls, which lead to the

formation of sclarenchymatous tissues, is also stopped following treatment of plant tops with maleic hydrazide.

Another fact of importance noted was that roots from treated plants were less pungent though sweeter in taste than those from controls. This is again explained as due to increased accumulation of sugars in roots of treated plants. Naylor (1951) observed a thirteen-fold increase in sugar concentration in corn when treated with maleic hydrazide. Large increases in sugar content in sugar beets have also been reported by Ririe and Mikkelsen (1952) and Wittwer and Hansen (1952), though no such increases were obtained by Stout (1952) and Peto *et al.* (1952).

Among the marked inhibition effects characteristic of MH treated plants may be cited the one on the development of bolting shoots. A marked suppression in scape development floral initiation, flower formation and fruit set is obvious. Change of flower colour to yellowish green and their quick abscission leading to premature flower and fruit drop are not uncommon. Other floral abnormalities, such as those in the development of petals, stamens, pollen grains, etc., also form notable effects of MH application. Peculiar effects in regard to pollen development seemingly result from a derangement in the meiotic division. Marked susceptibility of floral parts to MH application has been reported by White (1950), White *et al.* (1950) and Kennard *et al.* (1951) in raspberries and Choudhri and Bhatnagar (1953) in onions.

Male sterility in corn following MH treatment has been reported by Naylor and Davis (1950), Denisen and Haber (1950) and Josephson (1951). Eskew and Williard (1950) found that corn tassels were variously affected with different dosages of MH and pollen was produced but by the few florets that developed. In the case of millet these workers (1950) observed the formation of some seed heads but with number and size decreasing with increasing doses. Hoffman (1952) similarly reported lack of pollen production in corn following treatment with 0·10 M.H.

According to Curtis (1951) seed development was rare in *Lepidium* and *Barbarea vulgaris* when treated prior to flowering. Miller and Erskine (1949) and Chadwick *et al.* (1950) were able to prevent fruit development in Ginko through MH applications. Similarly, reduced seed pod development in milkweed was reported by Grigsby (1951).

It is interesting to note that whereas most growth regulators associate themselves with large increases in flower and fruit formation and better fruit set, maleic hydrazide behaves just the reverse. In this connection, the belief that a high auxin content is essential for proper flower development and fruit setting, appears correct. Maleic hydrazide, seemingly, behaves

as an auxin competitor (Leopold and Klein, 1951). It is reported to antagonize the effects of naphthalene acetic acid, 2, 4, 5-tri-chlorophenoxy-propionic and acetic acids (Smock *et al.*, 1952) and 2, 4-di-chloro-phenoxyacetic acid (Currier *et al.*, 1951; Hitchcock and Zimmerman, 1951; and Johnson and Buchholtz, 1951). Poor fruit set in radish plants in this investigation, therefore, seems to be a consequence of serious disturbances in the auxin content in the plants. According to Murneek (1939) insufficiency of hormone content leads to incomplete development or even suppression of flowers. Biddulph (1935), Murneek (1936–40), Harder *et al.* (1940) and Greulach (1948) reported the formation of the so-called ' vegetative' flowers, which are but anomalously developed flowers with morphological and histological variations following insufficient photoperiodic dosages leading to a disturbance in the hormonal balance of plants. Harder undertook extensive studies on the formation of inflorescences in *Kalanchæ blossfeldiana* (*a*) by limiting the number of effective photoperiods (short days); (*b*) by using intermediate photoperiods; (*c*) by interfering the transport of hormones by cutting the midribs of donor leaves; and (*d*) by variating the distance of transport of hormone. Under all the above procedures, reduced flower formation and transformation of inflorescences into vegetative branches were noticed. It seems clear, both, on the basis of the results obtained in this investigation as also on the evidence produced in the literature available that a serious disturbance in the hormonal balance of plants resulted following MH applications.

Treatment of floral shoots with maleic hydrazide has been found to induce an increased development of axillary buds. This is but natural, as a negation in the apical dominance would in the normal course bring about a development of axillary buds.

Treated radishes have been found to keep better during storage, even at as high a temperature as $21 \pm 1 \cdot 5°$ C. relative to controls which become pithy, hollow and even blackish within after a few days of storage. It is already explained as due to better water-holding capacity of roots from MH treated plants. This change in the quality character of radishes may also have resulted from other sided drifts in the physiological status of plants, *e.g.*, those in respiration, enzyme activity and the like. Isenburg *et al.* (1951) found that maleic hydrazide inhibited dehydrogenases, so important to plant respiration. Naylor and Davis (1951), likewise, reported an inhibition in respiration of onion root tips.

Summary and Conclusions

Knowing the fact that radish plants (*Raphanus sativus* L.) are quick flowering and radish roots, for which the crop is raised, become hard, woody

and unedible with the progress of bolting, the present enquiry was made in an effort to suppress or delay bolting to the advantage of vegetable growers. The plants for purposes of experimentation were grown on a fairly large scale under field conditions and maleic hydrazide used as a growth inhibitor. To achieve the end in view, 6-week old radish plants were sprayed with 0·05, 0·10 and 0·20 per cent. MH solutions with the help of a knapsac sprayer at the rate of 40 gallons to an acre. The following conclusions in regard to growth and bolting behaviour of plants have been arrived at:

Effects of MH spray were well visible after about a week of treatment, their intensity increasing with the age of the plants.

Fresh and dry weights of tops and roots were significantly affected by MH application. Maximum effectiveness of MH in roots, resulting in increased fresh and dry weights was shown by 0·10 per cent. aqueous sprays. As for tops, there was invariably a drop in the fresh weights and a rise in the dry. Highest dry weight was recorded following treatment with 0·10 per cent. MH.

Root/Top ratio was higher for all MH treated plants relative to controls both when computed on fresh and dry weight basis, 0·10 per cent. concentration being most effective in this respect. This is ascribed to a larger transport of food from tops to roots in the absence or delay in flowering.

With the formation of flowering shoot, the roots in the control plants became hollow, pithy or woody and entirely unedible, those for the unbolted treated plants remained fully turgid and edible for a long period of time.

Bolting in the treated plants was rare and that too greatly delayed, the intensity of inhibition depending upon the dosage of MH. The floral scapes when formed on the MH treated plants lacked adequate development.

The size of fruits, if formed on the treated plants, was small bearing fewer seeds. No fruit setting occurred in plants treated with 0·20 per cent. MH.

Several formative effects, *e.g.*, abscission of flower buds and metamorphic changes in the character of stamens and anthers, associated the treatment of plants with 0·20 per cent. MH. A negation in the apical dominance and an acceleration in the development of axillary branches were also characteristic of treated plants.

Radishes from treated plants were less pungent but sweeter in taste relative to controls. Furthermore, roots pulled out from field 1 week following treatment with 0·10 per cent. MH remained turgid and edible for a longer period while those from the untreated control plants readily lost their

turgidity and presented a shrivelled appearance. These latter ones when stored in this manner also become hollow and pithy within a few days time.

On the basis of the results of this investigation, use of MH sprays are recommended to the production of good quality 'radishes'.

Literature Cited

1. Barnard, E. E. and Warden, R. L. — "The effect of maleic hydrazide on various vegetable crops," *Proc. North Central Weed Control Conf., U.S.A.*, 1950, 145.
2. Biddulph, O. .. — "Histological variations in cosmos in relation to photo. periodism," *Bot. Gaz.*, 1935, **97**, 139–55.
3. Bonner, J. and Bandurski, R. S. — "Studies of the physiology, pharmacology and biochemistry, of the auxins," *Ann. Rev. Plant Physiol.*, 1952, **3**, 59–86.
4. Burleson, C. A. and Hubbard, J. L. .. — "Cotton defoliation in lower Rio Grande Valley," *Texas Agr. Exp. Sta. Progress Report*, 1397, 1951.
5. Cailahjan, M. H. .. — "Concerning the hormonal nature of plant development," *Comp. Rend. (Doklady) Acad. Sci. U.R.S.S.*, 1937, **16**, 227–30.
6. Chadwick, L. C., Miller, R. R. and Erskine, D. — "The prevention of fruit formation on some ornamental trees," *Proc. Amer. Soc. Hort. Sci.*, 1951, **58**, 308–12.
7. Cholodny, N. G. .. — "The internal factors of flowering," *Herb. Rev.*, 1939, **7**, 223–47.
8. Choudhri, R. S. and Bhatnagar, V. B. — "Prevention of premature bolting in onions following maleic hydrazide treatments," *Proc. Ind. Acad. Sci.*, 1953, **37B**, 14–21.
9. ———— .. — "Response of lettuce (*Lactuca sativa*, L.) to spraying with maleic hydrazide," 1953 (*Ref.* Zukel, J. W., Literature Summary on Maleic Hydrazide, *MHIS*, **6 A**, 7, March 1, 1953).
10. Clark, H. E. and Kerns, K. R. — "Control of flowering with phytohormones," *Science*, 1942, **95**, 536–37.
11. Cooper, W. C. .. — "Effect of growth substances on flowering of the pine apple under Florida conditions," *Proc. Amer. Soc. Hort. Sci.*, 1942, **41**, 93–98.
12. Crafts, A. S., Currier, H. B. and Day, B. E. — "Response of several crop plants and weeds to maleic hydrazide," *Hilgardia*, 1950, **20**, 57–80.
13. Currier, H. B., Day, B. E. and Crafts, A. S. — "Some effects of maleic hydrazide on plants," *Bot. Gaz.*, 1951, **112**, 272–80.
14. Curtis, O. F. .. — "Maleic hydrazide for grass control in fruit plantings," *Proc. North Eastern Weed Control Conf., U.S.A., Supplement*, 1951, 115–17.
15. Denisen, E. L. and Haber, E. S. — "Maleic hydrazide on sweet corn," *Proc. North Central Weed Control Conf., U.S.A.*, 1950, 147.
16. Dostal, von R. and Hosek, M. — "Uber der Einfluss von Hetero-auxin auf die Morphogenese bei Circaea (das Sachssche Phanomen)," *Flora, N.F.*, 1937, **31**, 263–86.
17. Eskew, E. B. and Willard, C. J. — "Maleic hydrazide on corn," *Proc. North Central Weed Control Conf., U.S.A.*, 1950, 187.

18. Eskew, E. B. and Willard, C. J. "Maleic hydrazide on soybeans, millet and rape," *Proc. North Central Weed Control Conf., U.S.A.*, 1950, 187.

19. Garner, W. W. and Allard, H. A. "Effect of relative length of day and night and other factors of environment on growth and reproduction in plants," *Jour. Agric. Res.*, 1920, **18**, 553–606.

20. Greulach, V. A. .. "Photoperiodic after-effects in six composites," *Bot. Gaz.*, 1942, **103**, 698–709.

21. Grigsby, B. H. .. "Reduction of seed formation in common milkweed, *Asclepias syriaca* L. by maleic hydrazide," *Proc. North Central Weed Control Conf., U.S.A.*, 1951, 142.

22. Hall, W. C. .. "The effect of sucrose and maleic hydrazide on the chemical defoliation and inhibition of second growth of cotton," *Texas Agr. Exp. Sta. Progress Report*, 1356, 1951.

23. Hammer, K. C. .. "Hormones and photoperiodism," *Cold Spring Harbor Symposia Quant. Biol.*, 1942, **10**, 49–59.

24. Harder, R. *et al.* .. *Jahrb. Wiss. Bot.*, 1942, **90**, 546–91 (*Ref.* Murneek, A. E., "History of Research in Photoperiodism," pp. 39–61, in Murneek, A. E. and Whyte, R. O., *et al.*, "Vernalization and Photoperiodism—A symposium," *Chronica Botanica*, Waltham Mass., 1948).

25. Hitchcock, A. E. and Zimmerman, P. W. "Absorption and movement of synthetic growth substances from soil as indicated by the response of aerial parts," *Contrib. Boyce Thompson Inst.*, 1935, **7**, 447–76.

26. ———— .. "A quantitative method of measuring response of plants to growth regulators," *Ibid.*, 1951, **16**, 225–48.

27. Hoffman, O. L. .. "Corn detasseling with maleic hydrazide" (*Ref.* Zukel, J. W., *Literature Summary on Maleic Hydrazide*, United States Rubber Coy., Naugatuck Chemical International Division, N.Y., U.S.A., 1952).

28. Hubbard, W. H. and Rowland, L. "Results of field plot experiment with maleic hydrazide in 1950 and 1951" (*Ref.* Zukel, J. W., *Literature Summary on Maleic Hydrazide*, United States Rubber Coy., Naugatuck Chemical International Division, N.Y., U.S.A., 1952).

29. Isenburg, F. M. R., Odland, M. D., Popp. H. W. and Jensen, C. O. "The effect of maleic hydrazide on certain dehydrogenases in tissues of onion plants," *Science*, 1951, **113**, 58–60.

30. Johnson, B. J. and Buchholtz, K. P. "Use of maleic hydrazide to antagonize proliferation and rooting of soybean stems induced by 2, 4-D," *Proc. North Central Weed Control Conf.*, U.S.A., 1951, 180.

31. Josephson, L. M. .. "Effect of maleic hydrazide in delaying flowering in corn," *Jour. Agron.*, 1951, **43**, 404–05.

32. Kennard, W. C., Tukey, L. D. and White, D. G. "Maleic hydrazide to delay blossoming of fruits," *Penn. State Coll., U.S.A., Progress Report No.* 52, 1951.

33. Kosar, W. F. and Thompson, R. C. "Some effects of maleic hydrazide on the growth of lettuce, 1951 (*Ref.* Zukel, J. W., *Literature Summary on Maleic Hydrazide*, United States Rubber Coy., Naugatuck Chemical International Division, N.Y., U.S.A., 1952).

34. Leopold, A. C. and Klein, W. H. "Maleic hydrazide as an antiauxin," *Science*, 1951, **114**, 9–10.

35. Lundegardh, H. .. "Environment and plant development" (Translated by Eric Ashby), Edward Arnold and Co., London, 1931.

36. Miller, R. R. and Erskine, D. "The prevention of fruit formation on some ornamental trees," *Proc. 25th National Shade Tree Conf.*, 1949, 88–89.

37. Murneek, A. E. .. 1936–40 (*Ref.* Murneek, A. E., "History of Research in Photoperiodism," pp. 39–61, in Murneek, A. E. and Whyte, R. O., *et al.*, "Vernalization and Photoperiodism—A Symposium", *Chronica Botanica*, Waltham, Mass., 1948).

38. ———— .. *Growth*, 1939, **3**, 295–315 (*Ref.* Murneek, A. E., "History of Research in Photoperiodism," pp. 39–61, in Murneek, A. E. and Whyte, R. O., *et al.*, "Vernalization and Photoperiodism—A Symposium," *Chronica Botanica*, Waltham, Mass., 1948).

39. ——, and Whyte, R. O., *et al.* "Vernalization and Photoperiodism," *Chronica Botanica*, Waltham, Massachusetts, 1948.

40. Naylor, A. W. and Davis, E. A. "Maleic hydrazide as a plant growth inhibitor," *Bot. Gaz.*, 1950, **112**, 112–26.

41. ———— .. "Respiration response of root tips to maleic hydrazide," *Bull. Torrey Bot. Club*, 1951, **78**, 73–79.

42. Naylor, A. W. .. "Accumulation of sucrose in maize following treatment with maleic hydrazide," *Arch. Biochem. and Biophysics*, 1951, **33**, 340–42.

43. Peto, F. H., *et al.* .. "Effects of pre-harvest sprays of maleic hydrazide on sugar beets," *Amer. Soc. Sugar Beet Technologists*, 1952, 4 (An Abstract).

44. Ririe, D. and Mikkelsen, D. S. "The effect of maleic hydrazide on sugar beet growth and sucrose content in certain field experiments," *Ibid.*, 1952, 3 (An Abstract).

45. Smock, R. M., *et al.* .. "Some effects of maleic hydrazide on the softening rate and respiration of apple fruits" (*Ref.* Zukel, J. W., *Literature Summary on Maleic Hydrazide*, United States Rubber Coy., Naugatuck Chemical International Division, N.Y., U.S.A., 1952).

46. Stout, M. .. "Two years results evaluating effects of preharvest sprays of maleic hydrazide on respiration and spoilage of sugar beets," *Amer. Soc. Sugar Beet Technologists*, 1952, 4 (An Abstract).

47. Thimann, K. V. and Lane, R. H. "After-effects of the treatments of seed with auxin," *Amer. Jour. Bot.*, 1938, **25**, 535–43.

48. Thurlow, J. and Bonner, J... "Inhibition of photoperiodic induction in Xanthium," *Amer. Jour. Bot.*, 1947, **34**, 603–04 (An Abstract).

49. van Overbeek, J. .. "Flower formation in pine apple as controlled by 2, 4-D and naphthaleneacetic acid," *Science*, 1945, **102**, 621.

50. White, D. G. .. "Blossoming of fruits delayed by maleic hydrazide," *Ibid.*, 1950, **111**, 303.

51. ———— , *et al.* .. "Maleic Hydrazide," *Amer. Fruit Grower*, 1950, **71**, 28.

52. Whyte, R. O. .. *Crop Production and Environment* Faber and Faber Ltd., London, 1946.

53. Wittwer, S. H., Coulter, L. L. and Carolus, R. L. "A chemical control of seed stalk development in celery," *Science*, 1947, **106**, 590.

54. ——— and Hansen, C. M. "Some effects of pre-harvest foliage sprays of maleic hydrazide on the sugar content and storage losses of sugar beets," *Amer. Soc. Sugar Beet Technologists*, 1952, 3 (An Abstract).

EXPLANATION TO FIGURES

FIG. 1. Radishes from the maleic hydrazide-treated and control plants exhibiting differences in size and in the production of lateral roots. They are also sliced longitudinally and transversely to indicate differences with regard to wood development. Whereas the radishes from control plants become woody, those from treated ones remain turgid.

A. Control,

a—a longitudinal slice.

a', transverse slice.

B. From 0·05 per cent. MH-treated plant,

C. From 0·10 per cent. MH-treated plant,

c', transverse slice,

D. From 0·20 per cent. MH-treated plant.

FIG. 2. One of those few plants which exhibited delayed flowering associated with several 'formative effects' following maleic hydrazide treatment of tops at 0·20 per cent. concentration. Stunted growth, fasciation, short internodes, and formation of ribbon-shaped leaves were characteristic of the floral shoot bearing very small buds. Consequent to these changes development of several axillary shoots was significant.

FIG. 3. A full-grown untreated radish plant photographed to show excessive development of the floral shoot bearing numerous flowers and fruits. Consequent to the translocation of large bulk of food the root remains less developed and becomes hard and excessively woody with age.

FIG. 4. A mature radish plant treated with 0·10 per cent. maleic hydrazide after the formation of young bolting shoot. Photograph shows a marked reduction in the development of the flowering shoot coupled with the formation of a number of axillary shoots. A dropping out of young buds and formation of seedless pods smaller in size relative to controls were characteristic.

FIG. 5. Roots from maleic hydrazide treated and control plants to exhibit differences in the keeping quality, photographed 3 weeks after storage.

A. From MH-treated plants; MH concentration 0·10 per cent.

a and *a'*, transverse and longitudinal slices to show retention of turgidity.

B. From control plants.

b and *b'*, longitudinal and transverse slices to show the shrivelled nature of roots; the interior became hollow, pithy and blackish.

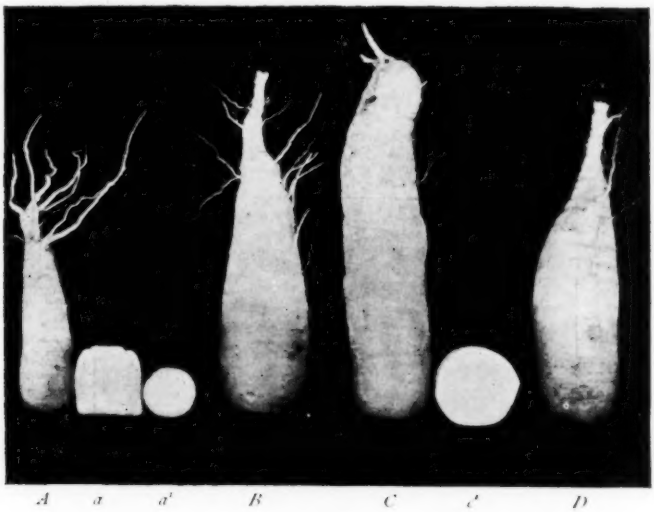


FIG. 1

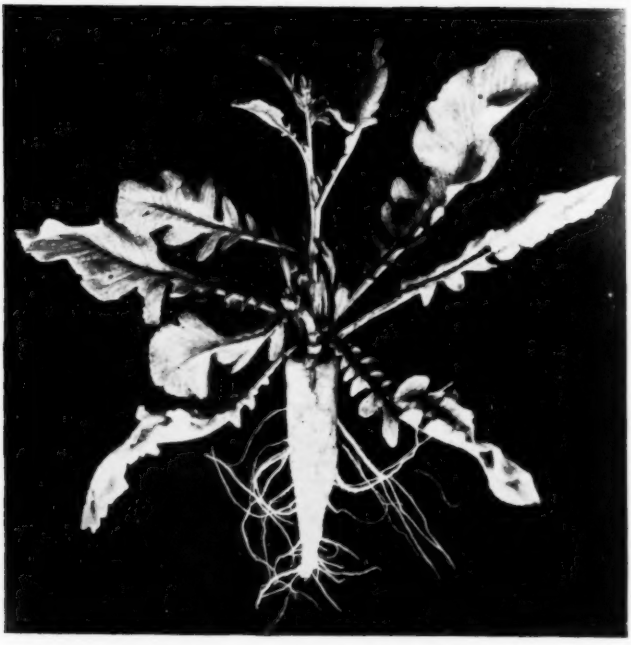
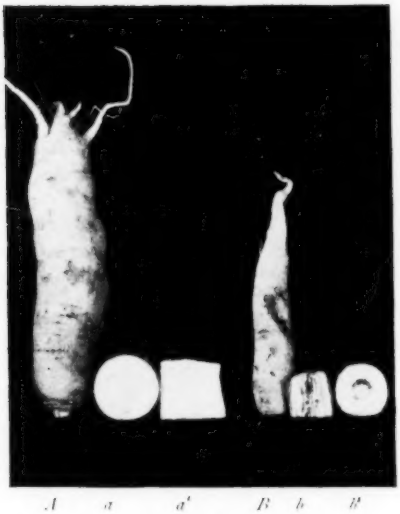


FIG. 2

FIG. 3

FIG. 4



A *a* *a'* *B* *b* *B'*

FIG. 5

STUDIES ON THE RED HALOPHILIC BACTERIA FROM SALTED FISH AND SALT*

By R. Venkataraman and A. Sreenivasan
(*Fisheries Technological Station, Kozhikode*)

Received June 9, 1953
(Communicated by Dr. B. S. Bhimachar, F.A.SC.)

Introduction

Preservation of fish by salting is as old as civilization and is still widely practised. A great deal of work has been done abroad on the bacteriology of salted fish, particularly on the halophiles. The terms " pink " or " pink eye " (Cloake, 1923; Shewan, 1937), " Rotwerdens " (Liebert and Deerns, 1930), "der rote Hund" (Horowitz-Wlassowa, 1931), " Red Heat " (Lochhead, 1934), " Rouge " (Bothello and Jose, 1949), " Rusty " (Harrison, 1923) and " Rote-erhitzung " (Stather, 1930), all refer to the same condition in salted fish or salted hides. The loss due to this affliction is incalculable.

Beckwith (1911) isolated *Diplococcus gadidarum* Beckwith from salted stock fish. Bitting (1911) in his extensive work noted that in all cases, a coccus, a bacillus and the cells of a fungus were associated with reddening, which he considered to be essentially a surface infection. The bacillus formed spores. Cobb (1927) found, that the infection was due to the solar salt, that salt from underground mines was not infected and that cold checks the growth of ' red ' bacteria. Kellerman (1915) studied the bacteria causing the red deterioration of salted cod fish and considered *Sarcina littoralis* (Poulsen), *D. gadidarum* (Beckwith), *Micrococcus litoralis* (Poulsen), and *Clathrocystis roseo percisina* (Farlow) to be synonymous. Harrison (1923) found *Pseudomonas salinaria* causing reddening of salted herring, while Gibbons (1937) isolated *Serratia salinaria*, *Ser. cutirubra* Lochhead, *Serratia* spp., *Bact. trapanicum* Petter, *S. littoralis* and *Sarcina* sp. from reddened salt fish. Horowitz-Wlassowa (1932) described *Tetracoccus carneus halophilus* which was later found to be identical with Petrova's strains and Patrova (1935) showed that the highly pleomorphic organism from red salted fish was identical with *Micrococcus roseus*. Liebert and Deerns (1930) isolated 3 different red microbes from brine, a coccus, a sarcina and a spore-forming bacterium. Shewan (1937) obtained from salted fish, a bacillus identical

* Published with the kind permission of the Director of Fisheries, Madras.

with Klebahn's *Bacillus halobus robur*, Harrison and Kennedy's *Ps. salinaria*, and Lochhead's *Ser. salinaria*, and a coccus related to *M. morhuæ*, Klebahn. Dixit and Vachha (1942) isolated a new strain, *Serratia sambharianus* from the salt lake Sambhar in Rajputana, India, which thrived even on dry salt. Similar isolation of *Ser. salinaria* from Aral sea region was made by Kurochkin and Emilyanchik (1937). Stuart *et al.* (1933) found that crude solar salts harboured red halophiles, while none of the 62 mined salts did. But the opposite view has recently been expressed by Mueller (1950).

Thus it is fairly clear that reddening of salted fish is essentially due to cocci and rod forms of bacteria originating from solar salts. We have isolated a number of such red halophilic organisms from various salts and salted fish and the results are presented in this paper.

MATERIALS, METHODS AND RESULTS

Isolations of red halophiles from salted fish were made by streaking the 'red' on plates of salt milk agar of Lochhead as modified by Hess (1942). Salt samples were plated out on the same medium after making decimal dilutions. This gave also the halophile count of the salts. A modified Gibbon's starch medium was also tried successfully for isolations from salts. Pure discrete colonies were subcultured on salt milk agar slants. The various media for studying the physiological and biochemical reactions were prepared according to Gibbons (1937) with the following exceptions: Potato slants were soaked for 48 hours in saturated brine and then sterilized at 15 lb. for 20 minutes. Starch hydrolysis was tested on salt milk agar plates incorporated with 0·2% B.D.H. soluble starch, after inoculation and incubation till good growth was obtained. All cultures were placed in a desiccator maintained at an R.H. of 75% with sulphuric acid, and incubated at 37° C. Gelatin tubes were incubated separately at the same temperature and after 7 days were periodically tested for liquefaction.

The following salt samples examined so far showed the presence of Red halophiles: Tuticorin salt, common salt, crushed from Tata Chemicals, Mithapur, Vedaranyam salt, and salt from the Model Farm at Wadala, Bombay.

Salted fish obtained over a wide geographical range also showed the 'Red' discolouration: Black pomfrets, white pomfrets, Mullets, *Leiognathus*, *Sillago*, catfish, *Polynemus*, *Therapon*, *Pristepoma*, the Dorab, and *Hemiramphus*, all from Rameswaram area, were afflicted by red discolouration profusely. So also were the catfish, *Polynemus*, Sharks and Skates received from Adirampatnam. On the West Coast, salted mackerels obtained from

Malpe, and salted soles, mackerels, silver bellies and sharks from Calicut market had an abundance of 'Pink' over the surface.

Washed and gutted fish salted in this laboratory with commercial Tuticorin salt for certain experiments (Venkataraman and Vasavan, 1953) were seen to develop the 'red'—Sharks, skates, mackerels, caranx, ribbon-fish, seer, silver bellies, the jew fish, soles and oil sardines, all developed the red in varying lengths of time.

Some of the organisms were obtained in pure cultures and a technical description of them is given below:—

Strains H_1, H_2 *and* H_{13}.—Colonies on salt milk agar, circular, red, moist viscid, glistening, entire; gram positive cocci in pairs mostly, in short chains and clusters, old cultures showing sarcinal tetrads; non-motile; liquefy gelatin; nitrites not produced from nitrates; indole not produced; H_2S not produced; starch not hydrolysed; dextrose, sucrose, lactose and xylose fermented with production of acid; casein digested with foul odour; red ring and pellicle in 20% NaCl broth; growth on potato, vermillion red, moist, viscid in less than a week.

Identity: *Sarcina* sp.

Source: From salted catla (Mettur) and salted sole.

Strain H_3.—Colonies bright red, circular, glistening, entire; gram positive cocci, single and pairs, non-motile; gelatin not liquefied; nitrates reduced to nitrites; indole and H_2S not produced; starch not hydrolysed; dextrose, sucrose and lactose not fermented; growth on potato raised, pink casein digested.

Identity: *Micrococcus* sp.

Source: Sole, salted in the laboratory.

Dr. Shewan (private communication) thinks this is not akin to any species described so far and that it reduces trimethylamine oxide to the amine.

Strain H_4.—Colonies red, circular, glistening, entire; gram positive cocci mostly single and pairs, a few in clusters; non-motile; gelatin not liquefied; nitrates reduced to nitrites; indole and H_2S not produced; starch hydrolysed; casein not digested; dextrose, sucrose and lactose not fermented; bright red circular colonies on salt potato.

Identity: *Micrococcus morrhuæ* Klebahn.

Source: Tuticorin salt.

Strain H_5.—Colonies orange-yellow to orange-red or pale-red, circular, glistening, entire; gram positive cocci mostly in pairs in salt milk agar and starch media but in packets of 4 or 8 in yeast extract agar; the diplococcus form appears to be motile when young; gelatin not liquefied; nitrates reduced to nitrites, indole produced; H_2S not produced; starch hydrolysed; dextrose, sucrose, lactose and xylose not fermented; casein not digested; on salt potato, flat orange-red streaks. Grows in KCl medium in place of NaCl.

Identity: *Micrococcus* sp.

Source: Salted mackerels.

Strains H_1, H_2 and H_{13} and also H_5 appear to us to be new species. Their exact systematic position is being examined by us and a report on it will be published later.

Strains H_6 *and* H_8.—Colonies vermillion red, circular, glistening, moist; gram positive cocci, single, pairs and packets of four; non-motile; gelatin not liquefied; nitrates not reduced to nitrites; indole and H_2S not produced; starch not hydrolysed; dextrose, sucrose and lactose not fermented; casein not digested; on salt potato, abundant wrinkled vermillion growth.

Identity: *Sarcina* sp.

Source.—Salted mackerels and sole.

Strain H_9.—Circular, reddish, glistening, moist colonies; gram positive cocci in pairs and tetrads, non-motile; gelatin not liquefied; nitrates reduced to nitrites; indole and H_2S not produced; starch not hydrolysed; dextrose, sucrose, lactose and xylose not fermented; casein not digested.

Source: Common salt.

Identity.—Corresponds to the description of *Sarcina littoralis* (Lochhead, 1934).

Strain H_{12}.—Colonies rose-red, moist, glistening, viscous; rods, non-motile, curved, single, pairs and short chains; gram variable, non-spore-forming, gelatin liquefied; nitrates reduced; indole produced from tryptone; H_2S not produced; starch hydrolysed; dextrose, sucrose, lactose and xylose not fermented; casein peptonized; on salt potato, raised, rose-red viscid growth, grows on 3·5% NaCl media.

Identity: Appears to agree with the description of *Serratia* species *b* (Gibbons, 1937) except for gram reaction.

Source: Vedaranyam salt.

It was observed that these red growths were viscous tending to draw out into fine threads on the needle, as experienced by Bitting (1911). Hence purification was rather difficult. In the salt milk agar medium, good growth covering the entire slant, or plate could be obtained in 7 days at 37° C., while at 4° C. there was no growth for the observed period of 4 months. At room temperature of 28 to 33° C. growth was slower than at 37° C. Incubation of the plates in the incubator without adjusting the humidity, resulted either in loss of moisture and dehydration of the medium, or absorption of too much of moisture, and growth was adversely affected. Cultures placed in a desiccator and maintained at an R.H. of 75% developed well. 0·1% acetic acid in the medium did not prevent the growth of these organisms.

Discussion

Though a great deal of work was done during the first three decades on the morphology of the halophilic bacteria, it was Lochhead's work (1934) that established the identity and synonimity of some of the confusing list of organisms described earlier. Later the excellent work of Shewan (1937, 1939) and Gibbons (1937) cleared the confusion in nomenclature and also yielded valuable information on the physiological properties. We came across only one rod form so far though many cocci were encountered both in salts and salted fish. Direct examination of the ' Red ' from the surface of afflicted salted fish, consistently showed the presence of three or four types of bacteria, *viz.*, gram positive sarcina; gram variable non-sporing rods; short, stout gram positive rods; and gram positive rods with terminal spaces, or " racket forms ". Shewan (1937) has mentioned that the bacillus with " terminal spaces " which look like spores may be either gas vacuoles or granules of sulphur. This is probable because when the ' red ' was streaked on salt milk agar, no sporeforming rods were ever encountered.

It was supposed that only rod forms, which were highly proteolytic and putrefactive, affected salted fish and hides. In fact Schoop (1935) detected no proteolytic activity on gelatin or in production of indole by these organisms. Our strains show that three of the cocci are gelatinolytic, while one produced indole. The above three strains in fact produced putrefactive odour in milk agar. At least four of our cultures peptonized milk showing their caseolytic power. Some of these, on salted fish, in course of time reduced the flesh to a pulpy mass. Their role in the spoilage of salted fish is thus evident.

As regards the effect of temperature, failure of growth at 4° C. noted by us agrees with the findings of Shewan (1939), Castell (1950) and Hess (1942).

A starch medium with salt, starch, beef infusion and tryptone was found to be much better than Lochhead's salt milk-agar, the growth manifesting itself in 48 hours in most of the cases. But chromogenesis was poorer and further work on this is in progress.

The susceptibility of various species of fish to the attack of 'Pink' may be widely different. Shewan (1939) has in fact found that " of the four species used Hake was the least susceptible ". We found the susceptibility in the following order in fish salted in the laboratory: Sharks and skates were covered with red in 19 to 30 days, mackerels after 30 days, caranx, ribbon fish and seer in 45 days and silver-bellies after three months, while oil sardines were seen to have the attack after four months. Salted fresh water fish like catla, labeo and catfishes also were susceptible. The variation in susceptibility of different species to red attack cannot be explained with any definiteness in the present state of our knowledge about these organisms.

SUMMARY

Red halophilic bacteria were isolated in pure culture from salted fish and solar salts. A description of one rod-shaped bacterium and a few cocci is given. Proteolytic tendencies were noticed both in the rod and in four of the cocci. While 37° C. and a relative humidity of 75% favoured good growth in laboratory media, at 4° C. there was no growth. Almost all the species of salted fish from Malpe, Calicut, Adirampatnam and Rameswaram, that were examined, developed red discolouration. Among the fishes salted in the laboratory, sharks and skates appeared to be susceptible to the attack earlier than the others and fatty fish like oil sardines developed it very late, if at all. The rod form was " trained " to grow in salt concentration of 3·5% while one coccus form grew in KCl medium in place of NaCl.

Use of sterilized salt for curing and storage at low temperatures are indicated for preservation of salted fish.

ACKNOWLEDGMENTS

Our grateful thanks are due to Dr. James M. Shewan, Torry Research Station, Aberdeen, for kindly checking the identity of 3 of our strains, and for helpful suggestions.

REFERENCES

Beckwith, T. D. .. "Ein Halophytischer Diplococcus," *Zentralblatt f. Bakt.*, II *Abt.*, 1911, **32**, 193–94.

Bitting, A. W. .. "Preparation of Cod and other salt fish for the market, including a bacteriological study of the causes of reddening," *U.S. Dept. Agric. Bur. Chem. Bull.*, 1911, **133**, 40.

Bothello, A. T. and Jose Freixo — "'Rouge' a red discolouration of cod fish," *Biol. Abstr.*, 1950, **24**, 33538.

Cobb, J. N. .. "Pacific Cod Fisheries," *U.S. Dept. Commerce Bur. Fish. Doc.*, 1927, **1014**, 457–58.

Cloake, P. C. .. "Red Discolouration (so-called 'Pink' or 'Pink eye') on dried salted fish," *Dept. Sci. Ind. Res.* (*Gt. Brit.*) *Food Invest. Bd. Special Rep.*, 1923, **18**.

Castell, C. H. .. "Storage temperature and control of red bacteria on fish," *Fish. Res. Bd.* (*Can.*) *Progr. Rep. Atlantic Coast Stas.*, 1950, **50**, 13–16.

Dixit, S. C. and Vachha, S. B. .. "*Serratia sambharianus*: A new species from salt lake of Rajputana," *Curr. Sci.*, 1942, **3**, 107–08.

Gibbons, N. E. .. "Studies on salt fish, I. Bacteria associated with the reddening of salt fish," *Jour. Fish. Res. Bd. Can.*, 1937, **3**, 70–76.

Harrison, F. C. .. "Rusty Herring," *Contr. Canad. Biol. Fish. N.S.* I, 1923, **13**, 279.

Hess, E. .. "Studies on salt fish, IX. Effect of environment on the growth of red halophilic bacteria, " *Fish. Jour. Res. Bd. Can.*, 1942, **6** (1), 10–16.

Horowitz-Wlassova, L. M. .. "Uber die Rotfarbung gesalzener Darme ('Der rote Hund')," *Zentralblatt. f. Bakt. II Abt.*, 1931, **85**, 12–18.

Kellerman, K. F. .. "Micrococci causing red deterioration of salted cod fish," *Ibid.*, 1915, **42**, 398–402.

Kurochkin, B. I. and Emilyanchik, K. B. — "The causative agent of red discolouration of salted fish of the Aral sea area," *Biol. Abstr.*, 1937, **12**, 70 (1938).

Liebert, F. and Deerns, W. M. .. "Uber die ursache des Rotwerdens von Pokelhering," *Zentralblatt. f. Bakt.* II *Abt.*, 1930, **80**, 33–35.

Lochhead, A. G. .. "Bacteriological studies on the red discolouration of salted hides," *Canad. J. Res.*, 1934, **10**, 275–86.

Müller, G. .. "Halophile Bakterien und Salzkonserven," *Food Sci. Abstr.* 1950, **24** (4), 304 (1952).

Petrova, E. K. .. "Etude sur le pleomorphisme de l' argent du rouge du poisson sale," *Biol. Abstr.*, 1935, **10**, 13924 (1936).

Schoop, G. .. "Obligate Halophile mikroben," *Zentralblatt. f. Bakt. Orig.* I *Abt.*, 1935, **134**, 14–26.

Shewan, J. M. .. "The bacteriology of 'pink' salted fish," *Dept. Sci. Ind. Res.* (*Gt. Brit.*) *Food Invest. Bd. Rept.*, 1936; (1937), pp. 108–09.

——— .. "The reddening of salt fish," *Ibid.*, 1938; (1939), 113–15.

Stuart, L. S., Frey, R. W. and James, L. H. — "Microbiological studies of salt in relation to the reddening of salted hides," *U.S. Dept. Agric. Tech. Bull.*, 1933, **383**.

Stather, F. .. " 'Rote verfarbung' und ' Rote Erhitzung' " auf gesalzener .. Roh-haute." Cited by Stuart *et al.* (*Supra.*)

Venkataraman, R. and Vasavan, A. G. — "Studies on the salting of marine fishes of Malabar Coast with special reference to the ratio of salt to be used," *Proc. Indian Sci. Congr.*, 1953 (Abstr.).

ON SOME METAMORPHOSING STAGES OF EELS (MURÆNIDÆ) FROM THE ESTUARY OF THE BURHABULONG RIVER, ORISSA STATE

By V. R. Pantulu and S. Jones

[*Central Inland Fisheries Research Station, Barrackpore (Calcutta)*]

Received May 28, 1953

(Communicated by Dr. H. Srinivasa Rao, F.A.SC.)

CONTENTS

	Page
Introduction	24
Muræna I—*Muræna* (*Gymnothorax*) *tile* (Hamilton)	25
Muræna II	30
Muræna III	32
General Remarks	34
Acknowledgment	35
Summary	35
References	35

Introduction

The present paper is a continuation of a recent account by the same authors (Jones and Pantulu, 1952) in which the metamorphosing stages of *Muræsox talabon* (Cantor.) and some Murænid leptocephali collected from some of the estuaries of Bengal and Orissa were described. From the collections made during the year 1952 in the Burhabulong estuary near Chandipore (Lat. 21·26; Long. 81·2) on the Balasore Coast, Orissa State, numerous leptocephali belonging to the genus *Muræna* Linnæus were obtained along with a number of juveniles of *Muræna tile* (Hamilton), *Pisoodonophis hijala* (Hamilton) and *Raitaboura raitaboura* (Hamilton) and the metamorphosing stages of *Muræsox talabon* (Cantor.) and *M. cinereus* (Forskal).

The Murænid leptocephali could be divided broadly into two groups a majority group of comparatively short specimens having 124 to 127 myotomes and the numerically insignificant group of relatively longer ones with 180 to 216 myotomes.

Amongst individuals of the first group, metamorphosing stages of three different species could be distinguished. There were some transitional stages of early eivers which could not be assigned with certainty to any of

the above three. A fairly connected series of stages from the toothed larva to an early elver, presumably of *Muræna tile*, belongs to one of the three species mentioned above. As the other two could not be referred to their respective adult forms they are described as Muræna II and III. The three species of *Muræna* described in this paper are different from the Murænid larvæ reported on by Deraniyagala (1934) and Nair (1948), in the number of myotomes and other characters.

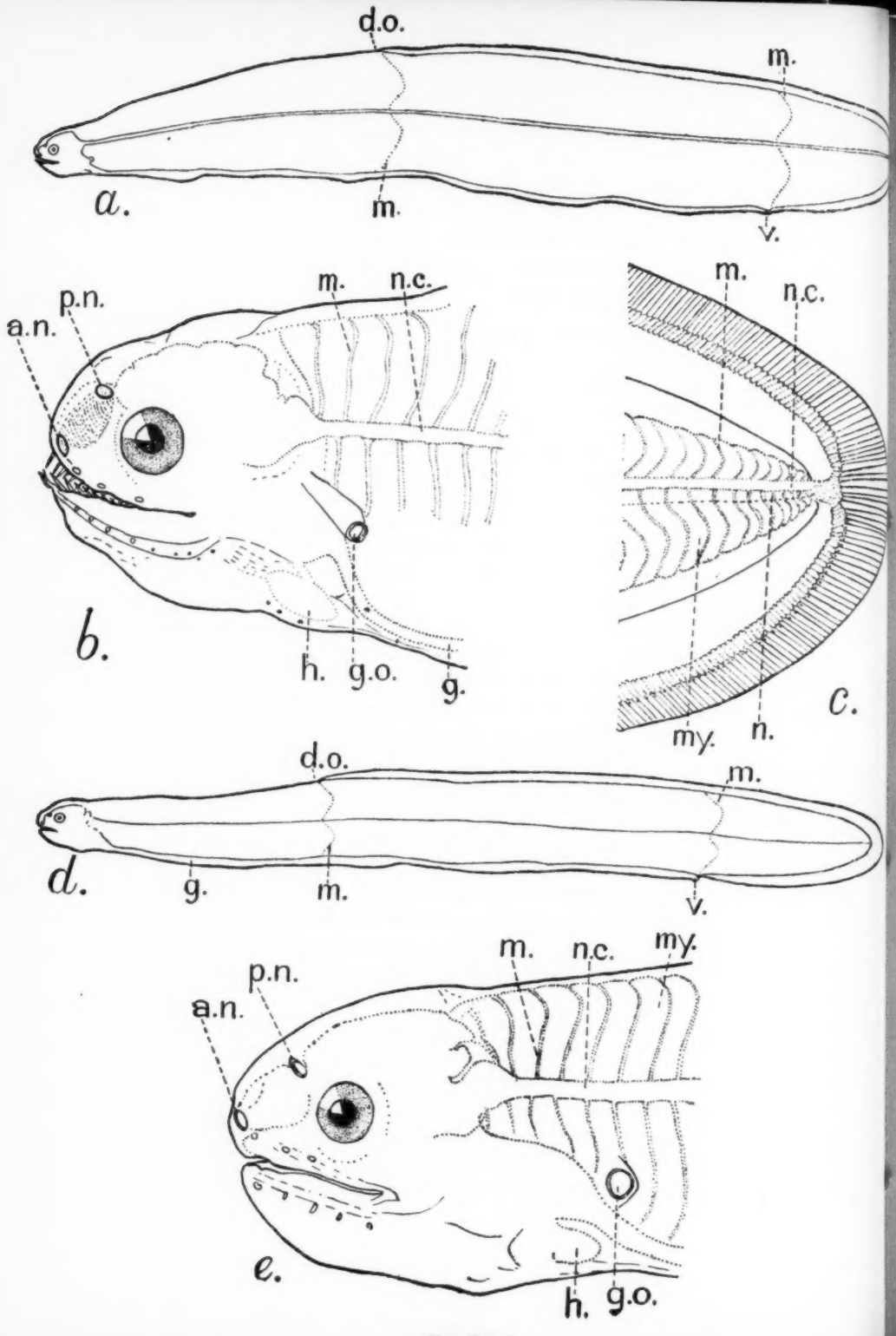
Only a few long specimens with 180–216 myotomes were obtained which, except for the difference in the myotome counts, were similar in general shape and appearance to the Leptocephali I, II and III described by the authors elsewhere (Jones and Pantulu, *op. cit.*).

MURÆNA I—*Muræna* (*Gymnothorax*) *tile* (Hamilton)

About 106 leptocephali of this kind ranging from a toothed leptocephalus to an early lightly pigmented elver were collected during the second and last weeks of June 1952, from the Burhabulong estuary near Chandipore. The main characters that place these leptocephali under *Muræna tile* are the number of myotomes which corresponds to the number of vertebræ in an adult *M. tile* and the general body proportions of the oldest metamorphosing stages in the collection. Further this species is the commonest Murænid in the estuaries of Bengal and Orissa, where it is known to ascend rivers more or less upto the tidal limit. The Leptocephalus IV provisionally assigned to *M. tile* by Jones and Pantulu (*op. cit.*) could now be definitely stated to belong to it.

Stage 1.—Total length 62·5 mm.; Myotomes 124 (Text-fig. 1 *a*, *b* and *c*).

The leptocephalus is transparent, ribbon-shaped, broad and rather short with a broadly rounded posterior end. The greatest depth of the body is 5·5 in the total length. The head which is nearly as broad as it is long is about 5 times the orbit and contained 15 times in the total length. The snout is blunt and is nearly equal in length to the orbit. The cleft of the mouth reaches a little behind the hind border of the eye. The upper jaw has on each side 12 larval teeth, the 5 anterior ones being larger than the posterior, which progressively decrease in size. The lower jaw has 10 teeth on each side, of which the 3 posterior ones are conspicuously small. The nostrils of each side are situated rather far apart, the posterior one above the eye, in a line with its front margin and the anterior one near the tip of the snout. A few pores are discernible along the lower margin of the upper jaw and along the sides of the lower jaw. Pectorals are absent, but a minute bud-like protuberance, present on each side immediately behind



TEXT-FIG. 1

TEXT-FIG. 1. Leptocephali of Muræna I, *Muræna* (*Gymnothorax*) *tile* (Hamilton). (*a*) Outline drawing of stage I, ×2·4; (*b*) Head of stage I, ×13·3; (*c*) Caudal end of stage I, × 13·3; (*d*) Outline drawing of stage II, × 2·4; (*e*) Head of stage II, × 13·3. *a.n.*, anterior naris; *d.o.*, dorsal fin origin; *g.*, gut; *g.o.*, gill opening; *h.*, heart; *m.*, myocomma; *my.*, myotome; *n.*, notochord; *n.c.*, nerve cord; *p.n.*, posterior naris; *v.*, vent.

the gill opening, seems to represent them. The dorsal originates opposite the 42nd myotome approximately midway between the snout and the vent and the anal just behind the vent between the 97 and 98th myotome. Both these fins are continuous with the caudal and the tail is nearly 5·5 in the total length.

Three chromatophores are present in the heart region and 5 chromatophores on the sides of the posterior half of the lower jaw. An irregular row of minute chromatophores is present along the base of the anal fin and along the ventral margin of the gut. A dendritic black chromatophore is found on either side below the gill slit.

Stage 2.—Total length 61·0 mm.; Myotomes 124 (Text-fig. 1 *d* and *e*).

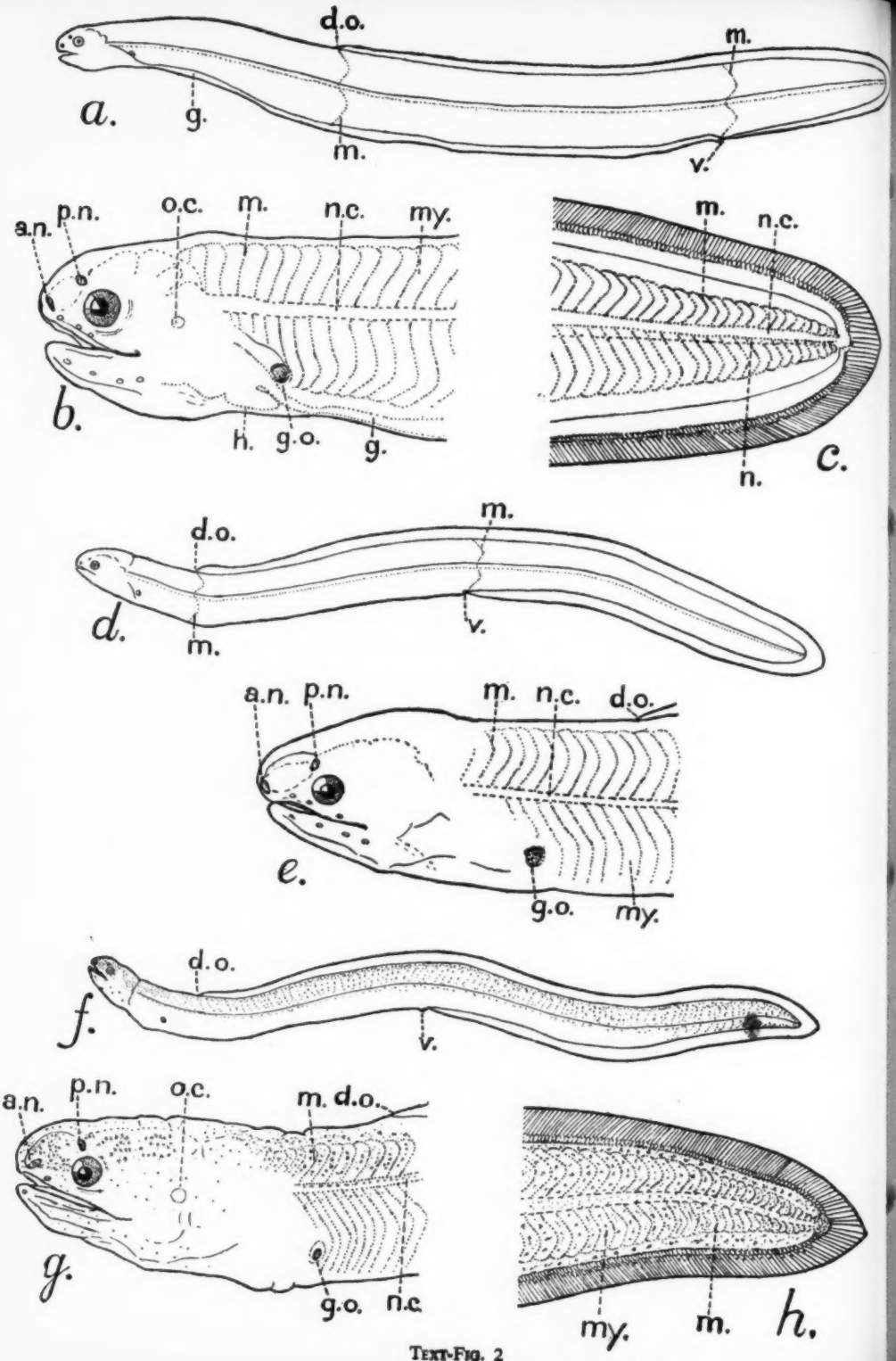
The leptocephalus is translucent and ribbon-shaped, but the depth of body which is now 8·5 in total length has perceptibly decreased. The decrease is rather abrupt in the region anterior to the dorsal origin. The head has grown in length and is 12 in the total length. The snout has become longer and is nearly 1½ times the orbit. All the larval teeth have already been shed. The vent has shifted forwards to the 39th myotome and the dorsal originates from the 92nd myotome. In addition to the chromatophores present in the previous stage a few more chromatophores appear along the middle region of the 6 posterior myocomma.

Stage 3.—Total length 60·0 mm.; Myotomes 124 (Text-fig. 2 *a*, *b* and *c*).

This is a very advanced translucent leptocephalus which is still flat and ribbon-shaped. A further decrease in height has taken place along the entire length of the body. The vent which has shifted still further anteriorly is situated opposite the 90th myotome. All the other body proportions remain almost the same as in the previous stage.

Stage 4.—Total length 55·0 mm.; Vertebræ 124 (Text-fig. 2 *d* and *e*).

This can be considered a transitional stage as the body has lost the characteristic flat ribbon-shaped appearance of a leptocephalus and assumed the cylindrical shape of an elver. The depth of the body is 12 in the total length. A further growth in the length of the head has taken place which is now 9 in the body. The eye appears to be smaller in size and is 8·5 in the head and 1·75 in the snout. The anterior nares have become more


a.
d.o.
g.
m.
m.
v.
b.
a.n.
p.n.
o.c.
m.
n.c.
my.
h.
g.o.
g.
c.
m.
n.c.
n.
d.
d.o.
m.
m.
v.
e.
a.n.
p.n.
m.
n.c.
d.o.
g.o.
my.
f.
d.o.
v.
g.
a.n.
p.n.
o.c.
m.
d.o.
g.o.
n.c.
h.
my.
m.

TEXT-FIG. 2

TEXT-FIG. 2. Leptocephalus and early elvers of *M. tile* (Ham.). (*a*) Outline drawing of stage III, ×2·4; (*b*) Head of stage III, ×8; (*c*) Caudal end of stage III, ×8; (*d*) Outline drawing of stage IV, ×2·4; (*e*) Head of stage IV, ×8; (*f*) Outline drawing of stage V, ×2·4; (*g*) Head of stage V, ×8; (*h*) Caudal end of stage V, ×8. *a.n.*, anterior naris; *d.o.*, dorsal fin origin; *g.*, gut; *g.o.*, gill opening; *h.*, heart; *m.*, myocomma; *my.*, myotome; *n.*, notochord; *n.c.*, nerve cord; *o.c.*, otocyst; *p.n.*, posterior naris; *v.*, vent.

tubulate. The dorsal has shifted further towards the anterior and is now situated opposite the 15th myotome, at a distance nearly equal to 3 orbit lengths behind the gill opening. The vent has shifted more towards the anterior, opposite the 64th myotome which marks almost the middle of the body. The gape of the mouth extends further and reaches well behind the posterior margin of the orbit. Indications of the formation of the adult set of teeth have begun to appear.

Stage 5.—Total length 53·0 mm.; Vertebræ 124 (Text-fig. 2*f*, *g* and *h*).

The larva has lost completely the characters of a leptocephalus and has become an elver. The head is 7 and the greatest depth is 13, in total length. The eye is 11 in the head and nearly 2 in the snout. The anetrior nares are tubulate while the posterior are less conspicuous. The vent and the dorsal have shifted still further anteriorly opposite the 58th and 12th myotomes respectively. The caudal fin is now pointed.

The already fairly pigmented body has now a relatively larger number of chromatophores than during the previous stage. A series of closely set chromatophores are discernible along the dorsal margin of the body and the lateral line. A few chromatophores are irregularly scattered on the dorsal half of the body along the myocomma. In the front and behind the eyes on each side close-set patches of chromatophores are present. Chromatophores are found on the base of the caudal fin.

Muræna I

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Total myotomes ..	124	124	124	124+ urostyle	124+ urostyle
Vent (myotome) ..	97/98	92	90	64	58
Dorsal origin (myotome) ..	42	39	39	15	12
Total length in mm.	62·5	61·0	60·0	55·0	53·0
Snout to vent ..	51·5	47·5	47·0	27·0	26·0
Snout to dorsal ..	25·0	21·0	19·0	8·30	8·0
Head ..	4·23	5·04	5·13	6·03	7·2
Snout ..	0·945	1·26	1·00	1·26	1·17
Eye dia. ..	0·9	0·81	0·765	0·72	0·63
Max. ht. (incl. fins) ..	11·61	7·20	6·75	4·5	4·05

The metamorphosis is almost complete and the body proportions chracteristic of *Muræna tile* are attained at this stage. As the elver grows into a juvenile the dorsal fin shifts a little further forward arising in front of the gill-openings and pigmentation of the body conforms to the pattern of the adult.

MURÆNA II

Examples of the second type of leptocephali, two representative stages of which are described in this section from a total of 49 specimens, were collected during the periods 8–10/6/52 and 23–27/6/52. The following characters help to differentiate this type of Leptocephali from that of *M. tile*: (1) presence of 126 myotomes as against 124 in *M. tile*; (2) the absence of chromatophores in the head region; (3) the difference in the number of larval teeth; (4) the more elongate shape of the body; and (5) the comparatively narrow and pointed head. It is probable that they belong to a species closely allied to *Muræna tile*, but it has not been possible to assign them to any particular species.

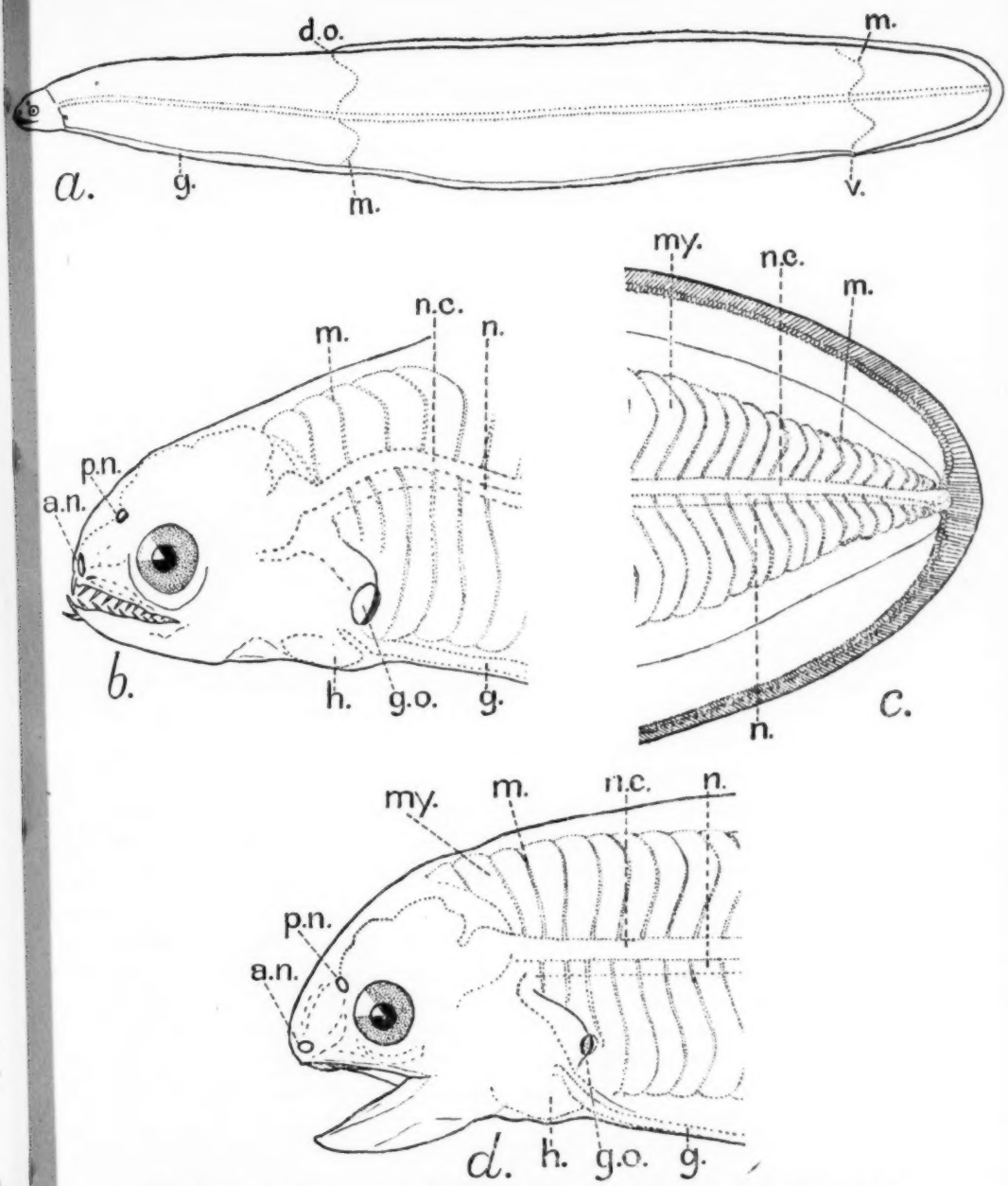
Stage 1.—Total length 68·0 mm.; Myotomes 126 (Text-fig. 3 *a*, *b* and *c*).

The leptocephalus is transparent, ribbon-shaped, with the snout somewhat pointed and the caudal end rounded. The greatest depth of the body is 7 in the total length. The head which is not as broad as in *Muræna tile* of about the same length is nearly 4 times the orbit and 19·5 in total length. The eye is nearly as long as the snout. The anterior nares are situated near the tip of the snout and the posterior slightly in advance of the anterior margin of the orbit, that is to say, nearer to the upper profile of the head than to the eye. Cleft of the mouth extends slightly beyond the middle of the orbit. The upper jaw has one median tooth at its tip and 16 on each side, the posterior ones being smaller than the anterior. The lower jaw has 12 teeth on each side with the two most anterior very prominent. Pectorals are represented by a very minute bud-like projection immediately behind each gill slit. The dorsal originates opposite the 43rd myotome nearer to the gill slit than to the vent, posterior to which arises the anal. The median fins are all continuous. The vent is situated below the 98th mytome and the tail is 6·2 in the total length.

No chromatophores are discernible in the head region. A row of irregularly arranged minute chromatophores are present along the base of anal fin and on the ventral side of the gut.

Stage 2.—Total length 56·0 mm.; Myotomes 126 (Text-fig. 3 *d*).

This is a slightly more advanced stage than the previous one. The depth of the body is now 8 and the length of head 15 in the total length. The snout



TEXT-FIG. 3. *Muræna II.* (*a*) Outline drawing of stage I, ×2·4; (*b*) Head of stage I, ×13·3; (*c*) Caudal end of stage I, × 13·3; (*d*) Head of stage II, × 13·3. *a.n.*, anterior naris; *d.o.*, dorsal fin origin; *g.*, gut; *g.o.*, gill opening; *h.*, heart; *m.*, myocomma; *my.*, myotome; *n.*, notochord; *n.c.*, nerve cord; *p.n.*, posterior naris; *v.*, vent.

is nearly as long or somewhat longer than the orbit. The eye is 4·5 in the head. The larval teeth have already been shed and the cleft of the mouth extends nearly upto the posterior border of the orbit. The vent is situated in the 94th myotome. The tail is 5 in the total length. The chromatophores are similar to those in the previous stage.

Muræna II

	Stage 1	Stage 2
Total myotomes	126	126
Vent (myotome)	98	94
Dorsal origin (myotome) ..	43	43
Total length in mm. ..	68·0	56·0
Snout to vent	57·0	45·5
Snout to dorsal	24·0	21·15
Head	3·51	3·69
Snout	0·90	0·945
Eye dia.	0·855	0·81
Max. ht. (incl. fins)	9·90	7·02

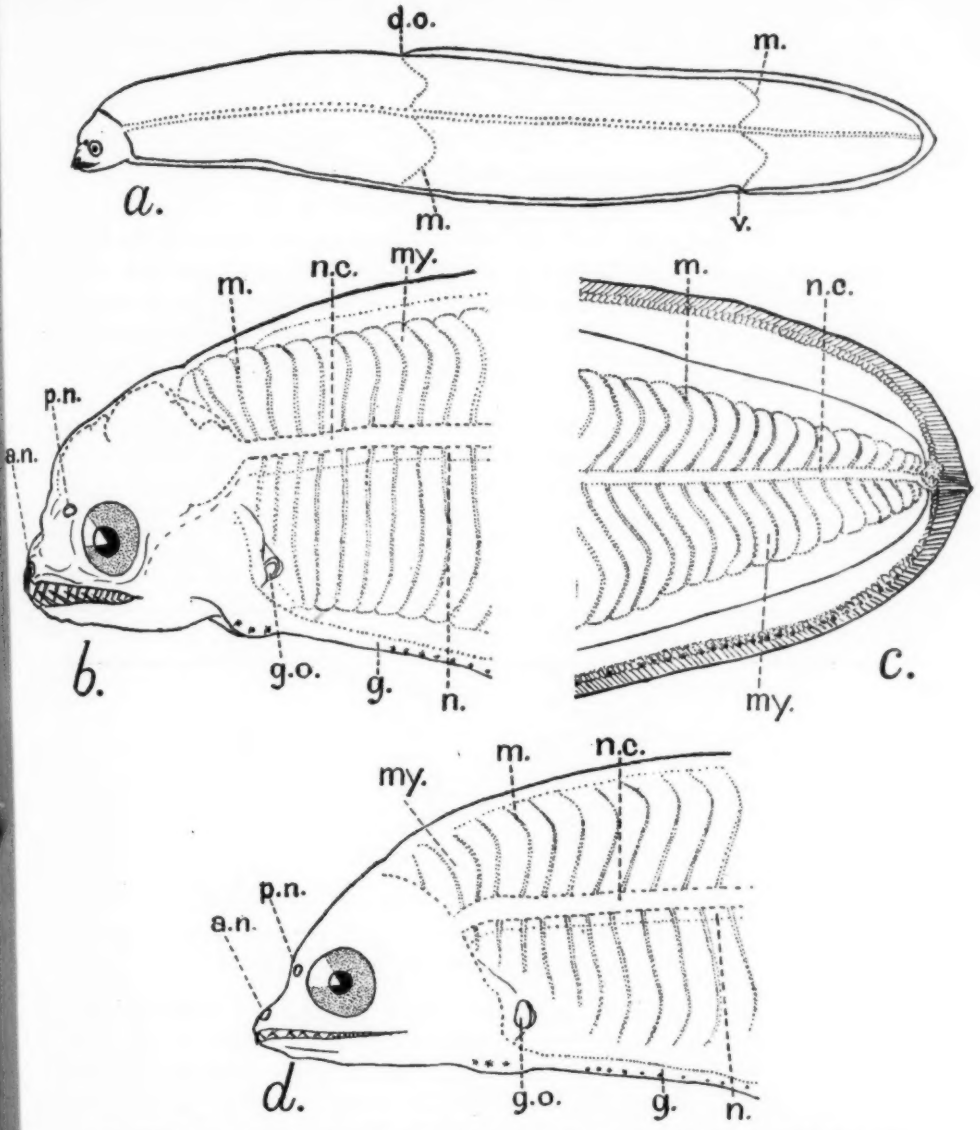
MURÆNA III

The third kind of leptocephali collected during 8–10/6/52 and 23–27/6/52 is distinct from the other two, described earlier, in its characteristic notched constriction above the tip of the snout, the larger size of the eye in relation to that of the snout and in the number of the larval teeth. There are 32 specimens of this series.

Notwithstanding these differences, the points of similarity that exist indicate that these leptocephali also belong to a closely allied species, but as advanced metamorphosing stages could not be obtained specific determination has not been possible.

Stage 1.—Total length 47·00 mm.; Myotomes 125 (Text-fig. 4 *a*, *b* and *c*).

The leptocephalus is transparent, short and ribbon-shaped with a blunt caudal end and a somewhat abruptly pointed snout. The snout has a characteristic conspicuous notch. The length of the head is 17 in the total length and the greatest depth of the body 5·5. The eye is very prominent and 3·75 in the head, and the snout is smaller than the eye. The anterior nares are situated at the tip of the snout and the posterior above the front border of the eyes. The cleft of the mouth reaches slightly behind the hind margin of the eye. Larval teeth are present. There are 17 in the upper jaw of which the anterior most is the most conspicuous, the rest progressively decrease in size towards the posterior end, those at the angle of the



TEXT-FIG. 4. *Muræna III.* (*a*) Outline drawing of stage I, ×2·7; (*b*) Head of stage I, ×13·3; (*c*) Caudal end of stage I, ×13·3; (*d*) Head of stage II, ×13·3. *a.n.*, anterior naris; *d.o.*, dorsal fin origin; *g.*, gut; *g.o.*, gill opening; *h.*, heart; *m.*, myocomma; *my.*, myotome; *n.*, notochord; *n.c.*, nerve cord; *o.c.*, otocyst; *p.n.*, posterior naris; *v.*, vent.

jaw being very minute. The lower jaw has 12 teeth of which the 7 anterior-most ones are larger than the rest. All the teeth are obliquely directed forwards and outwards. Pectorals are absent. The dorsal originates opposite the 38th myotome at a distance equal to 5 head lengths behind the gill slit and the anal fin commences immediately behind the vent at the 99/100 myotome. Both are continuous with the caudal. The caudal portion of the body is 3 in the preanal.

The head region is devoid of any chromatophores. Three chromatophores are present in the heart region, and a row of minute chromatophores ventral to the gut. A row of very small dot-like chromatophores is present along the base of the caudal fin.

Stage 2.—Total length 55·5 mm.; Myotomes 125 (Text-fig. 4 *d*).

This is an older stage than the previous one but metamorphosis has not yet commenced. The length of the head is 16·7 in the total length and the greatest depth of the body is 7·5. The eye which is larger than the snout is 4·5 in the head. The vent is below the 93rd myotome. Teeth are present.

Muræna III

	Stage 1	Stage 2
Total Myotomes	125	125
Vent (myotome)	99/100	93/94
Dorsal origin (myotome) ..	38	38
Total length in mm. ..	47·0	55·5
Snout to vent	37·5	45·5
Snout to dorsal	18·0	18·0
Head	2·97	3·33
Snout	0·765	0·72
Eye dia.	0·81	0·90
Max. ht. (incl. fins) ..	8·19	7·47

GENERAL REMARKS

The larval eels collected from the Burhabalong estuary were evidently brought in from the coastal waters with the tides. Absence of any early leptocephali in the collections made so far would seem to indicate that the breeding grounds of these eels may be far away and that the advanced leptocephali nearing metamorphosis are brought to the coastal and estuarine waters by strong currents. The present investigation though admittedly incomplete provides a link in the chain of the life-history studies of Indian eels.

ACKNOWLEDGMENT

Our thanks are due to Dr. H. Srinivasa Rao for critically going through this paper.

SUMMARY

Metamorphosing stages of 3 species of Murænids collected from the Chandipore, Burhabulong estuary (Orissa), are dealt with in this paper. The first species is provisionally determined as *Muræna* (*Gymnothorax*) *tile* (Hamilton) and a connected series of the above from a toothed leptocephalus of 62·5 mm. to an early elver of 53 mm. are described. In the absence of elver stages of the other 2 species their specific identification has not been possible, but from the fairly close similarity in characters they appear to be allied to the above species. The typical leptocephalus stages amongst them are described and figured.

REFERENCES

Deraniyagala, P. E. P. .. "Some Apodal Larvæ from Ceylon Waters," *Ceylon J. Sci.*, 1934, **19B** (1), 89–96.

Jones, S. and Pantulu, V. Rayappa "On the metamorphosing stages of the Talabon eel, *Muræne-sox talabon* (Cantor) with descriptions of some leptocephali from the estuaries of Bengal and Orissa," *J. Asiatic Soc.* (*Science*), 1952, **18** (2), 129–40.

Nair, R. Velappan .. "Leptocephali of the Gulf of Manaar," *Proc. Ind. Acad. Sci*, 1948, **27B** (4), 87–91.

2–54 Printed at the Bangalore Press, Bangalore City, by C. Vasudeva Rao, Superintendent
and Published by The Indian Academy of Sciences, Bangalore

STUDIES ON SOME ASPECTS OF BIOASSAY OF STOMACH POISONS

By S. Pradhan, Ph.D. (Lond.), D.Sc. (Luck.), F.R.E.S., F.E.S.I.
and
M. G. Jotwani, B.Sc. (Hons.), Assoc.I.A.R.I.
(*Division of Entomology, Indian Agricultural Research Institute, New Delhi*)

Received June 30, 1953
(Communicated by Dr. E. S. Narayanan, F.A.SC.)

Introduction

Recent discoveries of new synthetic insecticides like DDT, BHC and others which act both as stomach poison and as contact insecticide, have greatly widened the field where stomach poisons can be usefully employed for the control of mandibulate insect pests with biting and chewing habits.

While determining the median lethal doses of these insecticides as stomach poison for various pests one is faced with certain fundamental aspects which have to be thoroughly explored and understood before the bioassay of stomach poisons can attain the required standard of scientific precision.

In this contribution some light has been thrown on three of such aspects.

Material and Method

The required strengths of the insecticide used were prepared from the following chemicals:—

DDT.—p'p' isomer, prepared in laboratory from technical DDT by fractional crystallisation in rectified spirit.

Talc.—Super white grade, particle size 250/300 mesh, supplied by I.C.I. (India) Ltd.

Test Insect.—Third and fourth instar larvæ of *Euproctis lunata* Walk. reared in the laboratory were used.

Apparatus.—The dusting apparatus devised and perfected by Pradhan and Srivastava (1952) was used in all experiments for dusting leaf strips.

A plannimeter was improvised by getting specially prepared square paper photographed on a glass plate, one small square of this plannimeter being equal to 1·16 mm. square.

Method.—The well-known 'leaf sandwich' method of Campbell and Filmer (1929) was used with slight modifications.

Sandwiches were prepared as follows:—Leaf strips of required size from leaves of uniform age and thickness were cut and dusted along with weighed cover slips. Weight of the dust falling per unit area was determined by reweighing the dusted coverslips. Dusted leaf strips were covered with undusted leaf strips of the same size coated with a thin layer of freshly prepared strach paste. Sandwiches of required size were then cut from these leaf strip sandwiches. Each sandwich was mounted on a pin and fixed in a feeding dish, half filled with moist soil. The moist sand served the dual purpose of fixing the pin bearing sandwich and also maintaining enough humidity to keep the sandwich fresh. One weighed insect was confined in each of these dishes and allowed to feed overnight. Partially eaten sandwiches were removed next day, measured with the help of plannimeter and amount of poison eaten in milligrams per gm. body weight of the insect was determined. Insects thus fed with poisoned sandwiches were kept under observation at 24 hours' intervals for 6 days.

STARVATION AND SUSCEPTIBILITY

Three different batches of larvæ were starved for 3½ hours, 24 hours and 45 hours respectively and then fed (*a*) with sandwiches containing DDT and (*b*) with sandwiches containing only talc. The experiment was repeated with another set of 3 batches starved for 4 hours, 24 hours and 48 hours respectively. The temperature during these experiments varied between 106–12° F. The results of these experiments are given in Table I.

TABLE I

Period of starvation (Hours)	Average area (sq. mm.) of sandwich consumed per larva		Average dosage mg./gm.		Percentage kill in 6 days	
	DDT	Talc	DDT	Talc	DDT	Talc
			I. Experiment			
3½	8·2	26·5	·234	2·074	100%	Nil
24	7·7	22·5	·192	2·22	100%	,,
45	6·5	12·0	·121	0·975	50%	,,
			II. Experiment			
4	8·3	25	·238	2·78	87·5%	,,
24	7·5	25	·205	2·26	100%	,,
48	4·4	19	·123	1·74	60%	,,

As shown in Table I percentage mortality in larvæ starved for 3½ hours, 24 hours and 45 hours was 100%, 100% and 50% respectively. In the second experiment mortality in larvæ starved for 4 hours, 24 hours and 48 hours was 87·5%, 100% and 60% respectively.

Thus there is a decrease in mortality with increase in the period of starvation. These mortality data would lead us, at the first sight, to conclude that insect resistance increases with starvation. This conclusion is also supported by Munro's (1933) remark in the case of fumigation that the insect resistance increases with starvation. But, a closer study of Table I will show that the average amount of poison ingested by the larvæ which were starved for 45 or 48 hours and which gave a mortality of 50% or 60% is less than that of larvæ starved for 3½ or 4 hours and 24 hours which showed much higher mortality. Now the question arises whether this decrease in mortality in the larvæ starved for prolonged periods can be wholly explained by the decrease in the dose of poison ingested or there is some change in the insect resistance due to prolonged starvation.

Some light could be thrown on this point when we compared the results given in Table I with those obtained while determining the M.L.D. of the same stage larvæ after starving for 6 hours. These M.L.D. determinations, which are to be reported elsewhere in detail, were carried out when temperature ranged between 42–60° F. It was observed in these M.L.D. determinations that a dosage of 0·125 mg./gm. gave 62·5% mortality as against 60% mortality with a dosage of 0·123 mg./gm. after starvation of 48 hours at 106–12° F. as mentioned above. Now if we ignore the temperature factor, the results of two experiments, *i.e.*, 60% and 62·5% mortalities with dosages of 0·123 mg./gm. and 0·125 mg./gm. after 48 hours and 6 hours of starvation respectively, suggest that there was more or less equal resistance to the insecticide after 6 and 48 hours of starvation. But Ellisor and Blair (1940) and Dustan (1947) have shown that there is lower mortality at higher temperatures in the case of stomach poisons. Thus if the experiment for determining M.L.D. would have been carried out at 110–12° F., the mortality according to these authors would have been less than 62·5%. This line of argument tends to suggest a decrease in resistance with increase in period of starvation.

It appears that the prolonged periods of starvation, perhaps, make the *Euproctis lunata* larvæ somewhat lethargic resulting in feeding of comparatively smaller quantities of both poisoned and unpoisoned food. This is prominently shown by the larvæ starved for 45–48 hours when compared with those starved for 24 hours only. This reduced feeding naturally results

in reduced poisoning and lower mortality which apparently gives an impression of increase in resistance due to starvation.

It is possible that some expected effect of starvation, *i.e.*, increased feeding as a result of starvation might be observed if starvation periods were taken between 4 and 24 hours. However, Way (1949) in a preliminary experiment found that there was no increase in leaf area eaten after starvation for 3 hours, 13 hours and 24 hours, the leaf area consumed after these intervals being 1·3, 1·13 and 1·37 sq. mm. respectively.

BODY WEIGHT AND SUSCEPTIBILITY

Five different weight groups of *Euproctis* larvæ were fed on poisoned and unpoisoned sandwiches. From the area of sandwich eaten by each larva, the average area and the average amount of DDT or talc eaten by a larva in each group were calculated; this quantity of DDT or talc divided by average larval weight of the group gave the average dose consumed in mg./gm. body weight. These larvæ were kept under observations at 24 hours' intervals for 6 days.

TABLE II

Column	1	2		3		4	
Weight group	Average weight of larva (gm.)	Average area of sandwich consumed per larva (sq. mm.)		Average dosage consumed mg./gm.		Percentage kill after 6 days	
		DDT	Talc	DDT	Talc	DDT	Talc
a ..	·029	5·17	28·65	·1502	8·24	100%	Nil
b ..	·0427	8·93	32·48	·1745	5·1	85·7%	,,
c ..	·0607	11·48	31·56	·1578	4·33	71·4%	,,
d ..	·0732	12·29	31·9	·1401	3·63	60%	,,
e ..	·1081	25·87	31·56	·1996	2·43	60%	,,

There is a progressive decrease in the percentage mortality with the increase in weight of the larvæ upto weight group (*d*). This trend cannot be explained by any variation in the average dose consumed per gram body weight (Col. 3) by different weight groups. Of course there is no decrease in percentage mortality from weight group (*d*) to (*e*) but this may be explained by a much higher increase in the dosage consumed (·1996 mg./gm.) by weight group (*e*). Therefore it is indicated that the resistance of an insect to an insecticide increases with increase in body weight. Way (1949) also has

shown that when the dose per larva is the same, the mortality decreases with increase in body weight.

Column 2 shows that though the amount of poisoned food eaten increases with the increase in body weight, the dose or amount of poison ingested in mg./gm. body weight. (Col. 3) does not change in any definite order; it is in fact seen to be fairly constant in all the weight groups except the last one. This gives an indication that there is perhaps a constant amount of poison for unit body weight, which when ingested, causes the cessation of further feeding. This is further supported by the fact that the larvæ feeding on unpoisoned sandwiches have taken much more amount of sandwich when compared to the amount of poisoned sandwich eaten, suggesting that the poison produces some deterrent effects which compel the larvæ to stop feeding before fully satisfying their hunger.

No progressive increase in the amount of food eaten is perceptible in the case of sandwiches with talc only. This was observed only when the limited amount of sandwich (34·8 sq. mm.) was offered to them and it is possible that if unlimited amount of unpoisoned sandwich would have been given, the amount eaten would have been different in different weight groups.

Several Smaller Doses *Versus* One Large Dose

Three batches of larvæ fed on 3 sets of sandwiches each containing different amount of DDT per unit area (·000209, ·000384 and ·000597 mg. per sq. mm., Table III, Col. 1), and the fourth batch of larvæ acting as

Table III

Column	1	2	3				4	5	6
Set	Weight mg./ sq. mm.	No. of larvæ treated	Average area of sandwich eaten (sq. mm.) on four days				Average dose of first feeding mg./gm.	Average total dosage of 4 days mg./gm.	Percentage kill in 7 days
			1st	2nd	3rd	4th			
a ..	·000209	17	28·2 (14)	29·7 (12)	22·7 (10)	19·6 (10)	·0841	·20905	55·5%
b ..	·000384	19	17·8 (16)	20·7 (8)	15·7 (6)	22·7 (3)	·1035	·1952	89·4%
c ..	·000597	16	19·8 (11)	17·9 (5)	4·6 (1)	8·1 (1)	·2015	·2109	94%
Control talc ..	·000597	10	30·9 (9)	30·5 (9)	34·8 (10)	34·1 (9)	..	..	Nil

control was fed on sandwiches containing talc only. Feeding was carried out on 4 successive days to each group of larvæ.

The average area of sandwich eaten per larva of each group per day was calculated separately by summing up the area of sandwich eaten and dividing it by the number of larvæ which fed on that day (Table III, Col 3). For every group the average dosage for the first day of feeding was calculated by summing up the dosage of first feeding of all larvæ and dividing this by the number of larvæ which fed (Table III, Col. 4). The average total dosage of 4 days was calculated in similar way by summing the total dosage of 4 days of all larvæ in the group and dividing by number of larvæ treated in that group.

Figures in brackets (Col. 3) represent number of larva which ate sandwiches on that day. The successive decrease in the numbers is due to either death of the larvæ or their being affected by DDT.

Table III (Cols. 5 and 6) shows that the average amount of poison ingested during 4 days by all the 3 batches of larvæ, fed on sandwiches bearing different weights of poison per unit area, is more or less the same in all the 3 batches, but the mortalities (55·5%, 89·4% and 94%) vary considerably in the 3 groups. There is higher mortality in the batch fed on sandwiches bearing larger amount of poison per unit area. This is perhaps due to the fact that though the larvæ have ingested the same total dose, yet in case of larvæ fed on sandwiches bearing smaller amount of poison per unit area, the same total dose has been ingested in 2, 3 or 4 small instalments within 4 days, while the larvæ fed on sandwiches bearing larger amounts of poison per unit area the same dose has been ingested in a single instalment. This suggests that though DDT has a cumulative effect to a great extent, perhaps it is detoxified to some extent by the larvæ when it is taken in smaller doses in successive feedings. This argument is further strengthened by abnormal behaviour of some larvæ which survived total dosages of as high as ·355, ·342 and ·577 mg./gm., probably because these dosages were taken in 3 to 4 instalments during 4 days of treatment.

It is also seen from this experiment that the area of poisoned sandwich eaten decreases with increase in weight of poison per unit area, the decrease is more clearly seen in the total area of poisoned sandwich eaten in 4 days. But, though there is considerable decrease in area of sandwich eaten with increase in weight of poison per unit area, all the same the amount of poison ingested per gm. body weight on the first day of feeding increases with increase in amount of poison per unit area. This fact has direct bearing on the successive feeding on the poison by the same set of larvæ and it is seen that

the number of larvæ feeding on successive days is not only reduced from day to day but this reduction is quicker in case of larvæ fed on sandwiches bearing larger amount of poison per unit area (Col. 3).

Summary

Using DDT dust as insecticide and third and fourth instar larvæ of *Euproctis lunata* Walk. as test insect the following fundamental aspects of the bioassay of stomach poisons have been investigated.

Starvation and resistance.—It has been indicated that, contrary to previous notions, resistance of *E. lunata* larvæ decreases with increase in period of starvation. The apparent increase in resistance due to starvation is obviously due to the fact that prolonged periods of starvation (24–48 hours) make the *E. lunata* larvæ somewhat lethargic and these lethargic larvæ actually eat lesser quantity of both poisoned and unpoisoned food and therefore their percentage mortality is also low.

Body weight and resistance.—It has been shown that resistance to insecticide increases with increase in body weight. An increase in amount of poisoned sandwich eaten with increase in body weight has been observed, but the amount of poison ingested per gm. body weight of insect varied within a comparatively narrow range and showed no definite order of variation. It has been suggested that more or less constant amount of DDT per gm. body weight begins to have an inhibitory effect on further feeding.

Several smaller doses versus *one large dose.*—It has been shown that a comparatively larger dose given to larvæ in one instalment is more effective than the same dose given in several instalments of smaller doses. It is suggested that the detoxification of some of the poison may be the cause of this phenomenon.

Acknowledgments

The authors wish to record their gratitude to Dr. B. P. Pal, Director, and Dr. E. S. Narayanan, Head of the Division of Entomology, for providing all possible facilities for carrying out this piece of investigation.

References

Campbell, F. L. and Filmer, R. S. .. *Trans. 4th Int. Congr. Ent. Ithaca*, 1928, 1929, **2**, 523.

Dustan, G. G. .. *Canad. Ent.*, 1947, **79**, 1.

Ellisor, L. O. and Blair, C. R. .. *J. econ. Ent.*, 1940, **33**, 760.

Munro, J. W. .. *Agric. Live-stk. India*, 1937, **7**, 192.

Pradhan, S. and Srivastava, H. M. L. *Indian J. Ent.*, 1952, **13**, 209.

Way, M. J. .. *Ann. Appl. Biol.*, 1949, **36**, 86.

RELATION BETWEEN THE AGE OF RICE PLANT AND ITS SUSCEPTIBILITY TO HELMINTHOSPORIUM AND BLAST DISEASES

By S. Y. Padmanabhan and D. Ganguly
(*Central Rice Research Institute, Cuttack* 4, *Orissa*)

Received June 17, 1953
(Communicated by Shri R. L. M. Ghose, F.A.SC.)

Introduction

It has been reported that there were differences in the susceptibility of seedlings and mature plants of rice to Helminthosporium disease (*Annual Report, C.R.R.I.*, 1946–48). Such variations in the reaction of seedlings and mature plants have been noticed in the case of other plant diseases also (Lowther, 1951; Peturson, 1944; Tapke, 1953; and Tisdale *et al.*, 1926). Sherf *et al.* (1947) inoculated rice plants of different ages, from 15 to 85 days, with *Helminthosporium oryzæ* and observed that 15 days old plants had less infection than the older age groups tested. Between the latter groups there was no difference in infection. Suzuki (1940) noted that *Piricularia oryzæ* could infect the leaves of 25 days old seedlings but 3 months old plants remained unattacked. Anderson *et al.* (1947) reported that only plants in the seedling and tillering stages were susceptible to leaf infection due to Blast and that the resistance to leaf infection increased as the plants became older. This observation was confirmed by Hashioka (1950).

An understanding of the exact nature of relation between the age of the host and its susceptibility to a disease is of practical importance in carrying out varietal susceptibility tests under artificial conditions. It is necessary to know whether the reaction of the host, at the age in which it is tested, would serve as an index of its reaction throughout its life-history. Plants susceptible at seedling stage may become resistant as they become older and conversely, plants resistant at seedling stage may become more susceptible with increasing age.

The relation between the age of rice plant and its susceptibility to Helminthosporium and Blast diseases was studied in the present investigation. Both the pathogens, *Helminthosporium oryzæ* Breda de Haan [*Cochliobolus miyabeanus* (Ito et Kurib.) Drech. ex Dastur] and *Piricularia oryzæ* Cav. infect the leaves, leaf-sheaths, nodes, neck of the peduncle and grains

of rice plants. The studies in the present investigation were confined to foliar infection only.

Materials and Methods

Two different methods were adopted with respect to the two diseases. As Helminthosporium disease is sporadic in occurrence under natural conditions, artificial infection tests were carried out with plants raised in pots. On the other hand as Blast is of more general occurrence, reaction to this disease was studied under conditions of natural infection in field experiments.

Age of plants and susceptibility to Helminthosporium disease

Artificial infection tests were carried out for 2 seasons, 1949 and 1950 on three different dates in each year. By appropriate adjustment of the planting dates, plants of different ages were made available for inoculation on each date. Two susceptible varieties, Benibhog and T. 1145 were studied in 1949 and in 1950 a resistant variety T. 141 was also included. The intensity of infection was scored 7–10 days after each inoculation. The dates of inoculation, ages of plants infected and the intensities of infection are presented in Table I.

It may be seen from the data that:—

(i) In all the 3 varieties tested, the susceptibility of the plants increased with their age, *i.e.*, on any date of inoculation, the youngest plants showed the least infection, the infection increased progressively in plants of higher age groups and the oldest plants had the maximum infection.

(ii) Comparing different dates of inoculation, the September inoculation produced the highest amount of infection in plants of all ages and this was well marked in 45–50 and 92–95 days old plants in all the 3 varieties in 1950.

(iii) The relative susceptibility of the 3 varieties remained unaltered as compared between plants of the same ages, *i.e.*, when plants of the same age were compared on any date of inoculation, T. 141, which is a resistant variety, showed comparatively less infection than the susceptible varieties, T. 1145 and Benibhog.

Age of plants and susceptibility to Blast disease

The effect of the age of rice plants on their susceptibility to Blast disease was studied in field experiments during the year 1950 and 1951. In the first year the experiment was conducted with a moderately susceptible variety T. 1145 and in 1951 with a very susceptible variety Co. 13.

TABLE I

Intensity of Infection Developed on Plants of Different Ages at Different Parts of the Season

Age of plants in days	Date of inoculation	T. 1145			Benibhogh			T. 141		
		Average infection	Average No. of spots	Average size of spot in mm.	Average infection	Average No. of spots	Average size of spot in mm.	Average infection	Average No. of spots	Average size of spot in mm.
					1949					
19–22 days ..	30—7—1949	2·1	3	0·6×0·5	1·9	3	3·2×1·0			
	9—9—1949	4·5	8	2·0×1·0	3·4	5	1·8×1·0			
	3–11—1949 (Natural infection)	2·5	3	..	0·0	..	..			
49–57 days ..	30—7—1949	5·0	11	2·4×0·7	4·8	10	3·8×1·6			
	9—9—1949	4·0	7	2·6×1·0	3·7	5	1·8×1·3			
	3–11—1949 (Natural infection)	2·0	13	..	2·6	7	2·0×1·4			
94–96 days ..	3–11—1949 (Natural infection)	7·0	20	3·5×1·3	7·0	20	3·0×1·7			
					1950					
19–26 days ..	6—8—1950	2·8	1	1·8×0·7	2·5	1	1·6×0·6	2·4	1	0·5×0·4
	20—9—1950	2·9	1	Minute	2·8	1	Minute	3·8	1	Minute
	25–10—1950	2·6	2	Minute	3·4	5	1·6×1·6	2·0	2	1·0×2·0
45–50 days ..	6—8—1950	3·0	3	1·9×0·8	3·6	4	2·2×1·0	2·0	2	0·3×0·3
	20—9—1950	4·3	12	2·0×0·9	4·5	21	3·4×1·1	4·3	6	0·5×0·4
	25–10—1950	2·8	3	1·5×1·0	..	..	..	2·0	2	0·3×0·3
92–95 days ..	20—9—1950	7·0	80	3·1×1·0	7·0	64	5·2×2·4	4·7	16	1·0×0·4
	25–10—1950	4·2	12	3·5×1·0	5·0	9	4·0×1·5	3·0	4	3·0×1·5
127 days ..	25–10—1950	5·7	17	4·7×2·1	..	..	..	4·9	14	2·9×1·3

Experiment I.—In the experiment during 1950, the variety, T. 1145, was planted in two series, one with a basal dressing of green manure and the other with a top dressing of ammonium sulphate at the rate of 20 lb. of nitrogen per acre in addition to green manuring. In each series, there were 10 treatments representing 5 planting dates beginning from 20th July upto 29th August, with an interval of 10 days between 2 plantings. On each planting date, 30 and 40 days old seedlings were transplanted. These 10 treatments were randomised in 4 replications with individual plot size of 30 ft. × 5½ ft.

Though Blast can be noticed in the field under Cuttack conditions from July-November, the disease attains a peak period between the middle of September to middle of October. Observations on the foliar infection were taken on 29th September 1950 in the above experiment. On that date plants of 5 post-planting age groups, *i.e.*, 71, 61, 51, 41 and 31 days were available, half of the plants in each age group having been raised from 30 days and another half from 40 days old seedlings at the time of transplanting. The intensity of infection was quite uniform in plants within a plot. The incidence in a plot was estimated on the basis of a standardized scale. The mean infection data for plants of different age groups both under fertilized (green manure + ammonium sulphate) and unfertilized (green manure only) series are presented in Table II.

TABLE II

Showing Mean Leaf Infection Value per Plot in Variety T. 1145 *Observed on* 29–9–1950 *on Plants of Different Ages*

Manurial treatments	Age of seedlings at transplanting	Post planting ages in days				
		71	61	51	41	31
Green manure with Ammonium sulphate	30 days	1·50	2·25	3·88	11·38	16·12
	40 days	1·12	3·25	5·88	9·12	10·12
Green manure alone	30 days	1·00	1·25	1·75	5·25	7·12
	40 days	1·00	1·38	1·75	5·25	5·00

It may be seen from the data presented in Table II that the crop was progressively resistant to foliar infection due to Blast with increase in age and the extent of increase in infection from any one age to the next was highest between 51–41 days old plants. There was no effect of the 10 days difference in the ages of seedlings at the time of transplanting on the extent of infection. Only the post-planting age apparently determined the

susceptibility of the crop to the disease. The crop which had received additional manure as ammonium sulphate over the common basal dose of green manure had more infection than the one which did not receive ammonium sulphate.

Experiment II.—During 1951 the layout of the experiment conducted with a very susceptible early variety, Co. 13 was also in two series, one of which received no fertilizer while the second was fertilized with ammonium sulphate at the rate of 20 lb. N per acre. In each series, there were 4 planting dates beginning from 19th July 1951 with an interval of 10 days between 2 plantings. The treatments were randomized in 6 replications, the individual plot size being 30 ft. $\times 9\frac{1}{2}$ ft. Observations on foliar infection were taken on 14th September 1951, on which date plants of 4 post-planting ages, *i.e.*, 58, 48, 38 and 28 days were available, each having been raised from 30 days old seedlings at the time of transplanting. The observations were made on randomly selected sampling units in each plot. A plot was divided into 2 equal halves. From each half 2 random samples of 3 ft. $\times$ 3 ft. sq. were taken out and 4 corner plants from each square were observed. The intensity of infection was scored adopting the scale used in the previous experiment. The data representing the average infection value per plant are presented in Table III. The general prevalence of the disease was lower than in the previous year.

TABLE III

Showing Mean Infection Value per Plant in Variety Co. 13 *Observed on* 14–9–1951 *on Plants of Different Ages*

Manurial treatments	Post-planting age in days			
	58	48	38	28
Ammonium sulphate	2·45	3·77	4·78	4·81
No manure	1·32	2·78	4·11	3·53

It may be seen that in this experiment also the resistance of the crop increased with the increase in age of the plant. The plants which had received manure had more infection than the ones which had not received any.

DISCUSSION

The susceptibility of rice varieties of *Helminthosporium* disease increased with age, while the reverse was true in the case of Blast, within the ages studied. As has been stated earlier, this observation is of value in determining the

age at which the varietal susceptibility tests are to be conducted so as to get results of practical utility. Thus as far as the *Helminthosporium* disease is concerned, information obtained from seedling infection tests under optimum conditions of infection may be safely used for culling out the susceptible varieties, as with advancing age, these will become more and more susceptible. The varieties which come out as resistant in the seedling tests have to be tested further for their adult stage reaction, as resistance in the seedling stage may or may not be followed by the same in the adult stage.

The determination of the most susceptible stage of the host is also important for understanding serious outbreaks of the disease. Such epiphytotics develop only when the optimum conditions for infection and spread of the disease coincide with the susceptible stage of the host. It is therefore of particular significance that the two epiphytotics of the disease recorded hitherto in India, the Godavari Delta epiphytotic in 1918–19 (Sundararaman, 1922) and the Bengal epiphytotic in 1942 (Padmanabhan *et al.*, 1948) occurred when the crop was nearing maturity.

Regarding Blast disease, selections for disease resistance have to be carried out at the time of rapid growth following transplanting. At this age the plants are most susceptible. Varieties resistant at this stage, therefore, are likely to remain so at all other ages.

That even a difference of 10 days in the age of the crop results in a significant reduction of Blast incidence suggests that by suitably adjusting the planting dates, the severity of the disease and the loss caused by it could be minimised. It was stated that there is a peak period of Blast incidence in September-October period under Cuttack conditions. This peak period for Blast incidence varies from one locality to another depending upon the temperature, relative humidity, etc. A variety of short duration like Co. 13 which was used in one of the experiments may escape the severity of the disease in Cuttack by being transplanted sufficiently early so that the crop is in flower before the peak period of incidence sets in. This could of course be contemplated only under conditions of assured water-supply.

It is of interest to note that the changes that take place in rice leaf tissues as the plant attains maturity have opposite effects on the infectivity of the two pathogens, *Helminthosporium oryzæ* and *Piricularia oryzæ*.

Summary

The susceptibility of rice plants of different ages to *Helminthosporium* disease was studied by conducting artificial infection tests on three different dates during the season. Three varieties, 2 susceptible and a resistant were

included in the study. It was found that the susceptibility increased with age of plants and the relative susceptibility of varieties, as compared between plants of the same age remained the same at any time during the season. Inoculations carried out in September produced the maximum infection.

Susceptibility to Blast disease at different ages was studied in 2 field experiments. It was found that younger plants were most susceptible and the resistance increased as the plants grew older. Only the post-planting age of the plants had an effect on their susceptibility.

ACKNOWLEDGMENTS

The authors are indebted to Mr. T. P. Abraham, Statistician, for help in the statistical interpretation of the data, to Mr. K. Ramiah, formerly Director of Central Rice Research Institute and to Dr. N. Parthasarathy, Director, Central Rice Research Institute, for taking keen interest in the study and for many useful suggestions.

REFERENCES

Anderson, A. L., Henry, B. W. and Tullis, E. C. "Factors affecting infectivity, spread and persistence of *Piricularia oryzæ* Cav. *Phytopathology*, 1947, 37, **2**, 94–110.

Hashioka, Y. .. "Studies on the mechanism of prevalence of the rice blast disease in the tropics," *Tech. Bull. No.* 8, *Taiwan Agri. Res. Inst. Taipeh, China*, 1950, 80–84.

Lowther, C. V. .. "Seedling resistance *vs.* adult plant susceptibility of Mentana wheat to race 15 B of *Puccinia graminis tritici*," *U.S. Dept. Agr. Plant. Dis. Repr.*, 1951, **35**, 480–81.

Padmanabhan, S. Y., Raichoudhury, K. R. and Ganguly, D. "Helminthosporium disease of rice, I. Nature and extent of damage caused by the disease," *Indian Phytopathology*, 1948, **1**, 1, 34–47.

Peturson, B. .. "Adult plant resistance of some oat varieties to physiologic races of crown rust," *Canad. J. Res. C.* 1944, **22**: 287–89.

Sherf, A. F., Page, R. M., Tullis, E. C. and Morgan, T. L. "Studies on the factors affecting the infectivity of *Helminthosporium oryzæ*," *Phytopathology*, 1947, **37**, 281–90.

Suzuki, H. .. "Reaction of invasion of the blast flungus into rice plant to the difference of susceptibility of them," *Agri. and Hort.*, 1940, **15**, 1999–2010.

Tapke, V. F. .. "Further studies on Barley powdery mildew as influenced by environment," *Phytopathology*, 1953, **43**, 3, 162–66.

Tisdale, W. H. and Johnston, C. O. "A study of smut resistance in corn seedlings grown in the green house," *Jour. Agr. Res.* (U.S.), 1926, **32**, 649–68.

A CONTRIBUTION TO THE EMBRYOLOGY OF BOMBACACEÆ

By C. Venkata Rao

(Department of Botany, Andhra University, Waltair)

Received November 25, 1952

(Communicated by Prof. T. S. Sadasivan, F.A.SC.)

The Bombacaceæ with 20 genera and about 140 species (Willis, 1948) is treated by Bentham and Hooker (1862–83) as a tribe of Malvaceæ due to the presence of common characteristics like the large showy flowers, epicalyx, corolla with contorted æstivation and monothecous anthers. Engler and Prantl (1895), on the other hand, raised it to the rank of a family on account of the predominantly arborescent habit of its members and the presence of smooth-walled pollen grains. The present study was undertaken with a view to elucidate how far the embryological characters justify such a separation.

The previous embryological work in the family is meagre. Thirumalachar and Khan (1941) and Banerji (1942) studied the development of the female gametophyte and floss in *Eriodendron anfractuosum* DC. and *Bombax malabaricum* DC. respectively. Their observations can be summarised thus: The ovules in both species are crassinucellate, anatropous and bitegmic and show a zigzag micropyle. Usually a single hypodermal archesporial cell functions. Megaspore tetrads are linear in *Bombax* and T-shaped in *Eriodendron*. The chalazal megaspore functions and forms the 8-nucleate embryo-sac. The antipodals are ephemeral and in *Eriodendron*, they are said to degenerate even before the egg apparatus is organised. The polar nuclei fuse before fertilization. The case of an embryo-sac with reversed polarity was recorded in *Eriodendron anfractuosum*. In both species studied, the epidermal cells of the inside wall of the loculus develop into floss. In *Eriodendron*, the nucleus of the epidermal cell divides once mitotically before the cell begins to elongate.

There is no previous account of microsporogenesis, pollen grains, fertilization, embryo and seed development in the family.

This paper deals with the development and structure of the anther and pollen, ovule and embryo-sac in the following members of the family: *Eriodendron anfractuosum* DC., *Bombax malabaricum* DC., *Pachira aquatica* Aubl., *P. rosea*, *Adansonia digitata* L., *Ochroma lagopus* Sw. and fertilization,

developement of endosperm and embryo in the last named species. A few observations are also made on the seed development in *Adansonia digitata* and *Pachira aquatica*.

MATERIALS AND METHODS

The materials of *Bombax* and *Eriodendron* were obtained locally and those of *Pachira rosea* and *Adansonia digitata* were kindly sent by Miss S. Yesoda Devi, B.Sc. (Hons.), from Madras. The material of *Pachira aquatica* was sent by Mr. R. Seshagiri Rao from the Indian Botanic Gardens, Calcutta. A few trees of *Ochroma lagopus* (the balsa or cork wood) which is originally a native of S. America, are being successfully grown in Anakapalli Agriculture Farm, Visakhapatnam District, wherefrom the material was obtained. All materials were fixed in formalin-acetic-alcohol and the slides were stained in Heidenhain's or Delafield's hæmatoxylin or a combination of safranin and fast green.

FLOWERS

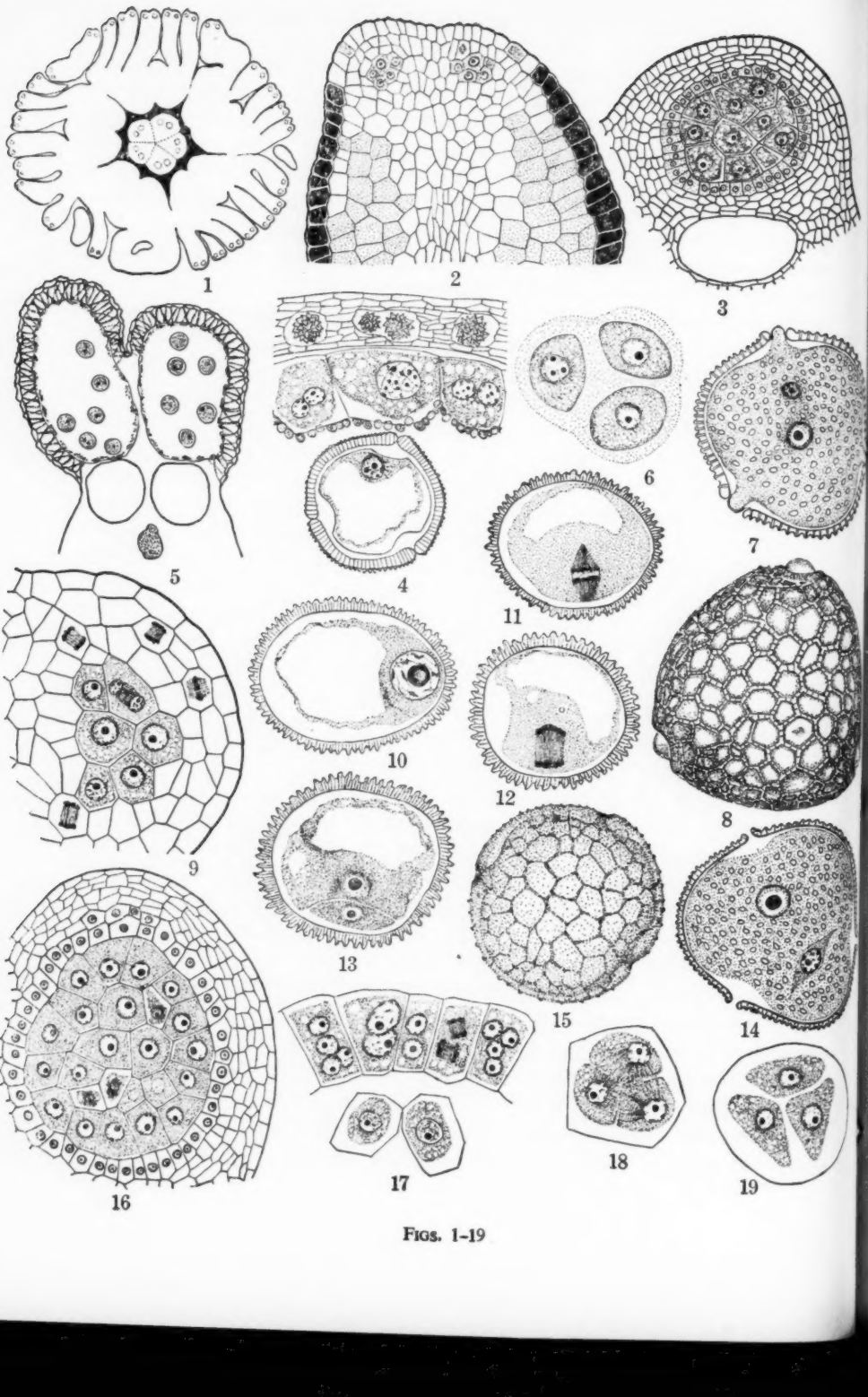
The flowers of all species are bisexual, pentamerous and dichlamydeous. The gynœcium is 5-carpellary except in *Adansonia* in which it is upto 8-carpellary (Fig. 53) and syncarpous. The septa do not fuse at the centre completely but leave a 5-angled space (Fig. 31). From the inner margins of the carpels arise the numerous ovules. The andrœcium shows much variation in different members. In *Eriodendron*, there are 5 large stamens with 2–3 thecous sinuous anthers. In *Ochroma*, there are 5 large stamens whose massive filaments twist spirally to form a column which surrounds the gynœcium. On the surface of this are found the numerous folds of the sinuous tranversely septate anther lobes (Fig. 1). In *Bombax*, there are 75 stamens in three series; the outermost of 5 antipetalous groups of 12 stamens each, a median ring of 10 stamens and the innermost of 5 stamens whose filaments and anthers are much larger than those of the rest and which are termed 'double stamens' (Gamble, 1935). In *Pachira* and *Adansonia* there are numerous stamens with renifrom monothecous anthers all of which are united at the base into a short tube.

MICROSPOROGENESIS AND MALE GAMETOPHYTE

The flowers in all species are protandrous, the formation of the microspore tetrads being completed by the time the megaspore mother cell is demarcated. One or two rows of hypodermal archesporial cells differentiate in the anther at two places. By a periclinal division these cells form the primary sporogenous cells to the inside and the primary parietal cells to the outside. Both sets of cells undergo repeated divisions, in all planes as a

result of which the anther wall becomes 5–7 layered and there is a great increase in the number of sporogenous cells, 12–20 being seen in a t.s. of the anther loculus (Figs. 3, 9, 16). This feature stands in sharp contrast with what usually obtains in Malvaceæ in which the primary sporogenous cells either function directly as the microspore mother cells or undergo very few mitotic divisions. The epidermal cells of the anther accumulate tannin from a very early stage, which stains deeply and diminishes with age (Figs. 2, 22, 26). Some cells of the connective also store tannin and starch grains. The sub-epidermal layer develops into fibrous endothecium (Fig. 5), and the innermost into the tapetum and the median layers ultimately get crushed. In *Ochroma lagopus* (Figs. 3, 5), the fold of the anther shows two loculi, below each of which a lysigenously formed mucilage sac develops. The sac enlarges with the growth of the anther and ultimately bursts as the cells between the anther loculus and the sac are crushed. The mucilaginous contents flow out and might assist in the attachment of the pollen grains to the body of the insect visitor. This device seems to be particularly useful because the pollen grains in this species are large as in Malvaceæ (measuring about 50 μ in diameter) but are devoid of spinescent outgrowths. Sometimes in *Ochroma*, some or all the microsporocytes in an anther loculus might degenerate; the loculus in such cases develops into a mucilage sac like the one below. A similar degeneration of all sporocytes in a loculus was met with in species of *Elæocarpus* (C. V. Rao, 1953). A number of druses were seen in the cells of the wall between the loculi in *Ochroma* (Fig. 4), similar to those in *Triumfetta rhomboidea* (C. V. Rao and K. V. S. Rao, 1952), *Helicteres isora* and *Thespesia populnea*. Whether they are of any use to the plants or are only products of metabolism, is not clear. Druses are also reported in the placentæ of cotton (Iyengar, 1938).

In all species studied, the tapetum was found to be of the secretory type. As divisions in both the cells of the anther wall and sporogenous cells continue for a long time, the tapetum is organised rather late in the development of the anther (Fig. 16). It is biseriate at places, which seems to be associated with the nutrition of the large number of microsporocytes in a loculus. As usual, the tapetal cells become binucleate in the early prophase I of the sporocytes. Due to further free nuclear divisions, some of the cells become 3–4 nucleate, the latter condition being more common in *Bombax* (Fig. 17). By a fusion of the nuclei again, a single large lobed polyploid nucleus is sometimes formed (Fig. 4). In *Ochroma*, cutin in the shape of beads is deposited on the inner walls of the tapetal cells (Fig. 4). These become prominent by about the time of formation of microspore tetrads but are seen even in the degenerating tapetal cells. A similar condition



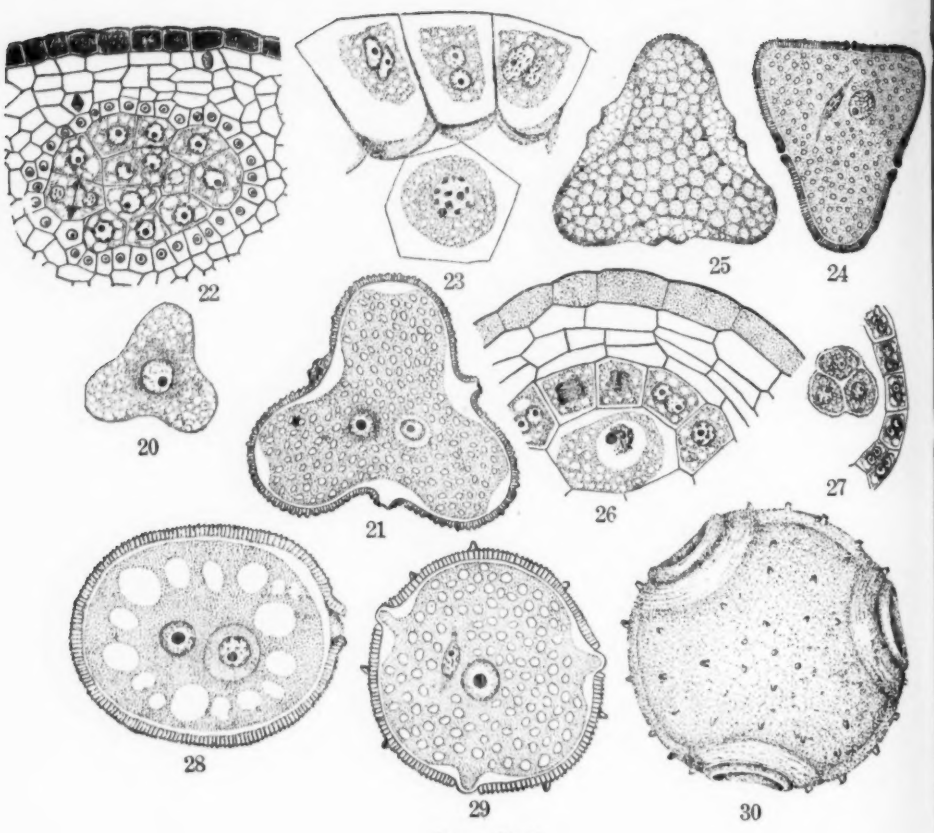
FIGS. 1–19

FIGS. 1–19. Microsporogenesis and development of the male gametophyte in Bombacaceæ. Figs. 1–8. *Ochroma lagopus*. Fig. 1. T.s. staminal tube, ×135. Fig. 2. T.s. anther lobe, ×200. Fig. 3. T.s. older anther lobe showing organisation of tapetum and mucilage cavity, ×165. Fig. 4. Part of the anther showing druses, tapetal cells with cutinised inner walls and a 1-nucleate pollen grain; note secondary 1-nucleate condition of tapetal cell, ×425. Fig. 5. T.s. mature anther showing fibrous endothecium, pollen grains and degenerate tapetal cells, ×45. Fig. 6. Young microspore tetrad surrounded by special wall, ×715. Figs. 7 and 8. Sectional and surface views of mature pollen grains, ×425 and ×500 respectively. Figs. 9–15. *Eriodendron anfractuosum*. Fig. 9. T.s. young anther lobe showing divisions in parietal and sporogenous cells, ×500. Figs. 10–13. Stages in development of male gametophyte, ×715. Figs. 14 and 15. Sectional and surface views of mature pollen grains, ×425. Figs. 16–19. *Bombax malabaricum*. Fig. 16. T.s. anther showing organisation of tapetum; note degenerating microsporocytes, ×285. Fig. 17. Tapetal cells and sporocytes, ×715. Fig. 18. Four-nucleate microsporocyte showing cytokinesis, ×715. Fig. 19. Young pollen tetrad invested by special wall, ×715.

was described by Kosmath (1927) in *Magnolia youlum* and *Lilium tigrinum*. In *Pachira* species, the inner walls of the tapetal cells become mucilaginous and swell (Fig. 23), but the protoplasts, as in other species, remain *in situ* and degenerate gradually. In showing the secretory type of anther tapetum, Bombacaceæ resemble closely Sterculiaceæ, Elæocarpaceæ and Tiliaceæ and differ widely from Malvaceæ in which it is uniformly of the plasmodial type.

A number of microsporocytes degenerate even in the resting condition (Fig. 16). Probably this ensures better nutrition to the functional ones. The two meiotic divisions proceed normally with a distinct interphase. The microspores are commonly arranged in a tetrahedral manner (Figs. 6, 19, 27), bilateral tetrads being rare. The protoplasts of the sporocytes round off in prophase I but their cell walls persist till about the time of formation of microspore tetrads, as L. N. Rao (1941) also noticed in *Hibiscus trionum*. The protoplasts get covered by a callose sheath (Fig. 6). Cytokinesis occurs by furrowing which commences even before the disappearance of the spindle fibres in the tetranucleate pollen mother cell (Fig. 18). The callose wall follows the deepening furrow and finally the wedges meet at the centre encircling each microspore. In due course, the sheath gelatinises liberating the young microspores which do not as yet show the rudiments of the exine (Fig. 20). So L. N. Rao's (1941) suggestion that in *Hibiscus trionum* the callose sheath is used up in development of the spore wall does not hold good in Bombacaceæ.

The nucleus of the microspore divides while its protoplast is in the shape of a lining layer, forming a lenticular generative cell (Fig. 13). The metaphase and anaphase spindle in *Eriodendron anfractuosum* was found to be asymmetrical with blunt generative and pointed vegetative poles (Fig. 11), though the telophase spindle appears normal (Fig. 12). Brumfield



FIGS. 20–30

FIGS. 20–30. Microsporogenesis and development of the male gametophyte in Bombacaceæ. Fig. 20. Young microspore of *Bombax malabaricum*, ×715. Fig. 21. Two-nucleate pollen grain of *Bombax*; note generative cytoplasm, ×715. Figs. 22–24. *Pachira aquatica*. Fig. 22. T.s. anther lobe, ×330. Fig. 23. Tapetal cells and sporocyte; note mucilaginous inner walls of tapetal cells, ×425. Fig. 24. Sectional view of 2-nucleate pollen grain, ×425. Fig. 25. Surface view of mature pollen grain of *Pachira rosea*, ×425. Figs. 26–30. *Adansonia digitata*. Fig. 26. T.s. anther lobe, ×425. Fig. 27. Tapetal cells and young microspore tetrad, ×350. Fig. 28. Young pollen grain; note generative cytoplasm, ×715. Figs. 29–30. Sectional and surface views of mature pollen grains, ×500.

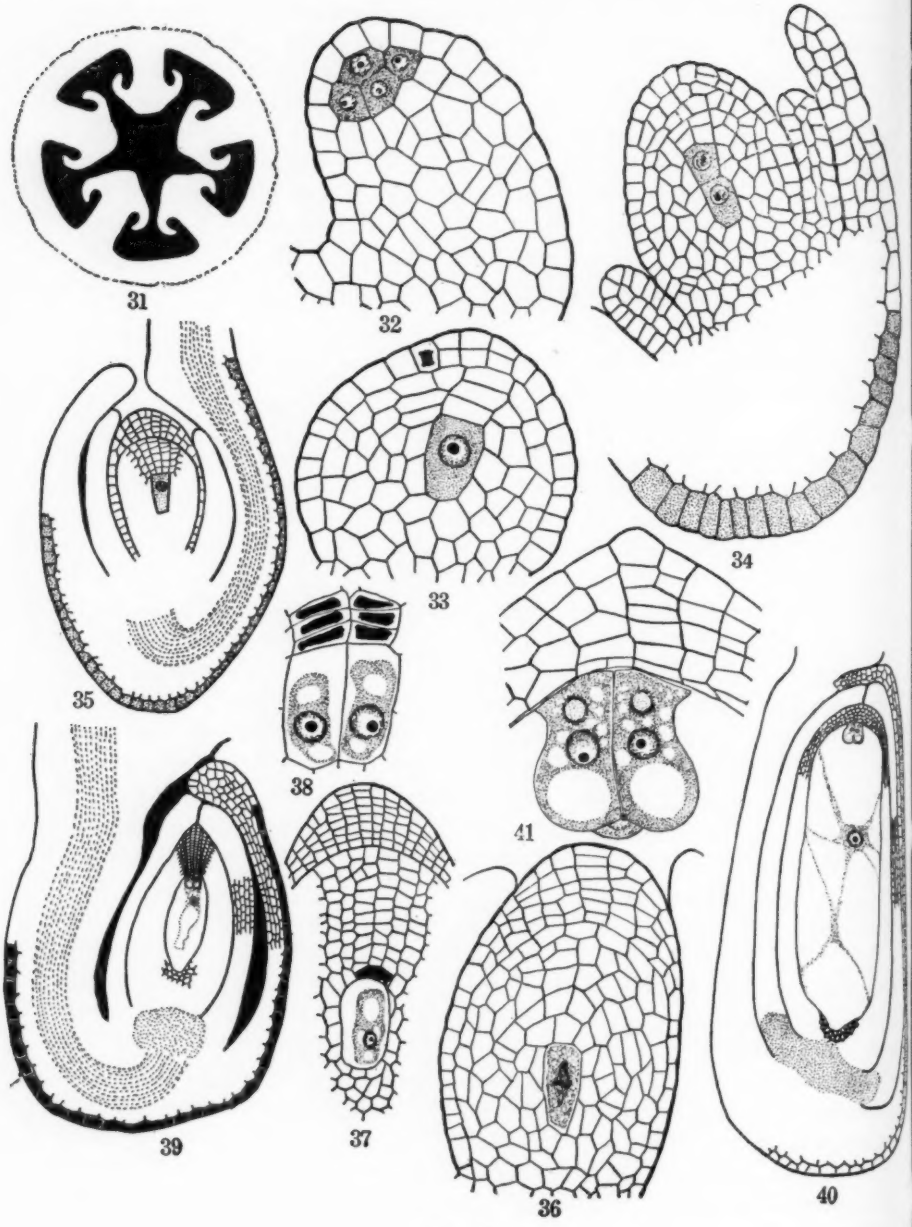
(1941) correlates the asymmetry of the spindle with the flattened shape of the prophasic nucleus. But since in this case, the prophasic nucleus was perfectly normal and spherical (Fig. 10), the asymmetry seems to be due to the scanty amount of cytoplasm found towards the generative pole as was also found in *Triumfetta rhomboidea* (C. V. Rao and K. V. S. Rao, 1952). The sheath of generative cytoplasm remains distinct around the generative

nucleus for some time after the cell has migrated into the vegetative cytoplasm (Figs. 21, 28), but later it becomes obscured by the large number of starch grains that accumulate in the vegetative cytoplasm (Fig. 7). The generative cytoplasm is brought out clearly in preparations stained in Delafield's hæmatoxylin. In mature pollen grains, the generative nucleus becomes ellipsoidal and its two ends are capped by relatively deep staining conical zones of cytoplasm (Figs. 14, 24, 29), as was also noticed in the pollen grains of *Triumfetta* (C. V. Rao and K. V. S. Rao, 1952). The pollen grains are shed in this two-nucleate stage (Figs. 7, 21). Degenerating pollen grains with normal exine but scanty or no cytoplasm, were commonly noticed in all species.

The pollen grains in the different species are of two shapes: in *Adansonia*, *Eriodendron* and *Ochroma*, they are spherical (Figs. 8, 15, 30), and in *Pachira* and *Bombax*, they are triangular and oblately flattened (Figs. 21, 25). The latter resemble the pollen grains of the Helictereæ of the Sterculiaceæ (C. V. Rao, 1950), though the position of the germ pores is different. In Helictereæ, they are situated at the angles while in *Bombax* and *Pachira* they are found at the middle of the sides. In all but *Adansonia*, the surface of the exine shows reticulate pattern (Figs. 8, 15, 25). In *Adansonia* there are small knob-like protuberances. The rim of the germ pore shows a collar-like thickening (Fig. 30) and there is a ring-like harmomegathus around the germ pore which appears closely similar to that seen in Dombeyeæ of Sterculiaceæ (C. V. Rao, 1950). The intine is uniformly thin except around the germ pore (Fig. 7). In all species, the pollen grains are triporate though a few in *Ochroma* are 4-porate. In external features the pollen grains of Bombacaceæ resemble the smooth-walled triporate pollen grains of Sterculiaceæ, Elæocarpaceæ, and Tiliaceæ and differ markedly from those of Malvaceæ which are characteristically spinescent and multiporate.

Ovule

The ovules arise as small conical papillæ on the inner margins of the carpels even before the latter have met at the centre of the ovary (Fig. 31). In *Adansonia* they are found on thread-like structures which arise in a row on each carpellary margin (Figs. 53, 54) and appear somewhat circinotropus in the young condition. The mature ovules are anatropous, bitegmic and crassinucellate (Figs. 39, 40, 44, 47, 64, 79). The initials for both the integuments arise simultaneously (Fig. 70). As in other Malvales, the outer integument is thinner and grows faster than the inner. It covers up the nucellus even before the megaspore mother cell has divided (Figs. 35, 74). Except in *Pachira aquatica* in which it is 5-layered, it is 2–3



FIGS. 31–41

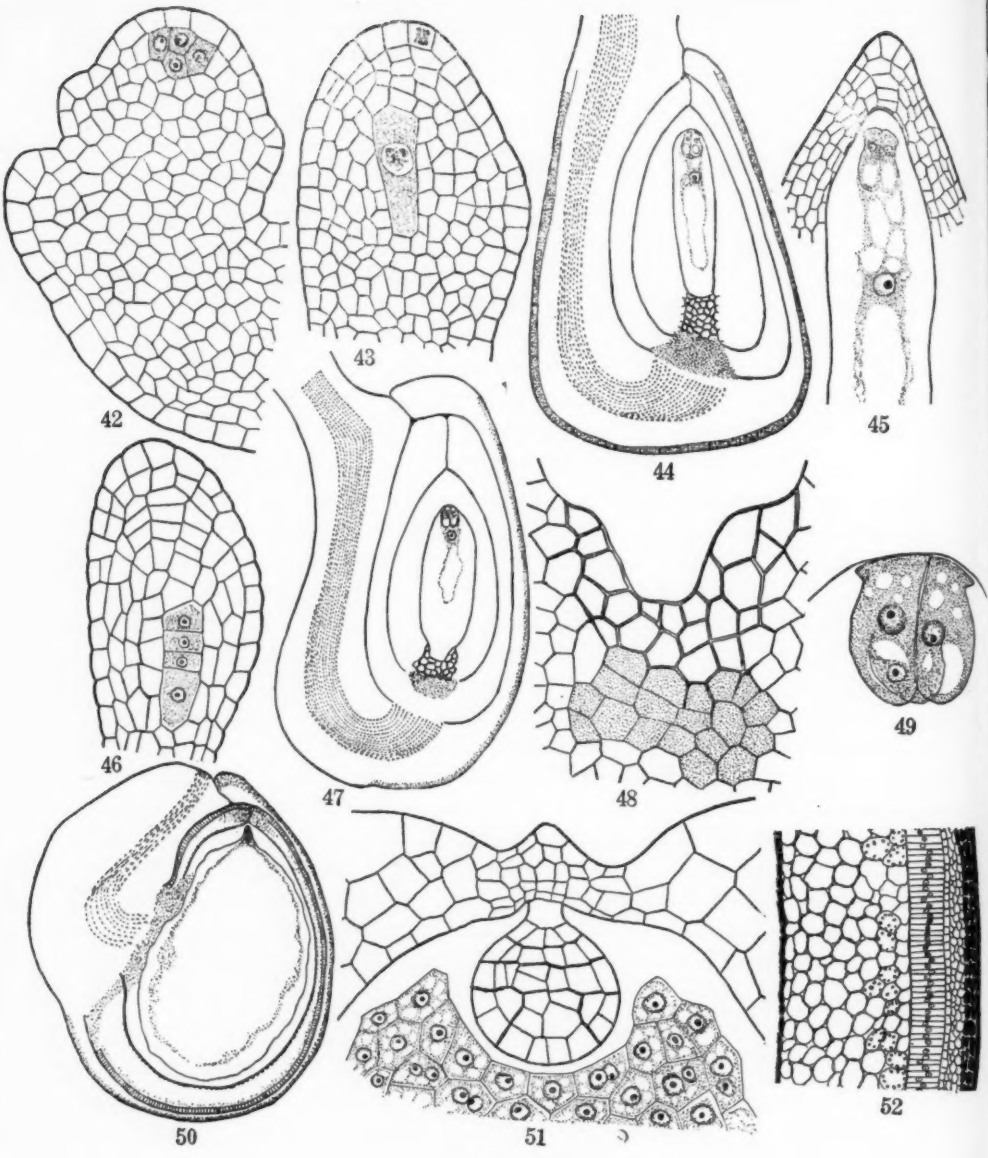
FIGS. 31–39. *Pachira aquatica.* Fig. 31. T.s. ovary with ovule primordia, ×30. Fig. 32. Ovule primordium with multicellular archesporium, ×425. Fig. 33. Nucellus showing formation of parietal tissue and nucellar cap, ×425. Fig. 34. Young ovule with 2 functional megaspore mother cells, ×500. Fig. 35. Ovule with full grown megaspore mother cell, × 135. Fig. 36. Meiosis I in megaspore mother cell, ×200. Fig. 37. Nucellus showing 1-nucleate embryo-sac, ×200. Fig. 38. Two tetrads from an ovule, ×425. Fig. 39. Mature ovule, ×75. FIGS. 40 and 41. *Pachira rosea.* Fig. 40. L.s. fertilisable ovule, ×75. Fig. 41. Egg apparatus; note ring-shaped patches near the micropylar ends of synergids, ×425.

cells thick. The cells of the outer epidermis of the outer integument accumulate tannin early in the development of the ovule (Fig. 34). The inner integument is 4–7 layered and unlike in members of Sterculiaceæ, Elæocarpaceæ and Tiliaceæ all cells remain normal and richly protoplasmic without accumulating tannin in the fertilisable ovules; tannin is, however, accumulated in some cells after fertilization (Fig. 50). The micropyle has the zigzag form characteristic of the Malvales (Figs. 39, 40, 47, 64, 79). An air space develops between the integuments commonly (Figs. 39, 79).

The nucellus is straight and massive. By the time the megaspore mother cell prepares for meiotic divisions, it is covered by 8–12 layers of parietal tissue and nucellar cap. The latter is relatively thicker in *Pachira* and *Adansonia.* Some of the parietal cells later get crushed by the enlarging embryo-sac and 4–10 layers of cells stand above the mature embryo-sac. There are 5–8 layers of cells around the embryo-sac and as many below it. Two or more layers of cells around the lower end of the sac become thick-walled as in Sterculiaceæ, Elæocarpaceæ and Tiliaceæ (Figs. 39, 40, 44, 48, 63, 80). Consequently the sac shows the characteristic shape with broad micropylar and narrow antipodal ends. The chalazal region of fertilisable ovule stands out conspicuously due to the presence of deep staining tannin-filled cells (Fig. 40). The ovules of *Pachira rosea* are more elongated than those of *P. aquatica* (Figs. 39, 40). While in *Adansonia, Eriodendron* and *Bombax* they are conical, in *Ochroma* they look top-shaped due to the presence of a chalazal outgrowth (Fig. 92). In *Ochroma lagopus*, some of the ovules in a loculus become malformed. In these, the integuments fail to cover the nucellus completely and the nucellus is also relatively scanty (Figs. 84, 85).

MEGASPOROGENESIS AND EMBRYO-SAC

The primary archesporium of the ovule consists of a group of hypodermal and sub-hypodermal cells (Figs. 32, 42, 71). Its differentiation synchronises with the demarcation of the integument initials. Usually only one cell functions and the rest merge into the nucellus. Occasionally, however, more than one cell were seen to function till the megaspore mother cell (Figs. 34, 72) or the tetrad stage (Fig. 38). In such cases, they were placed



FIGS. 42–52

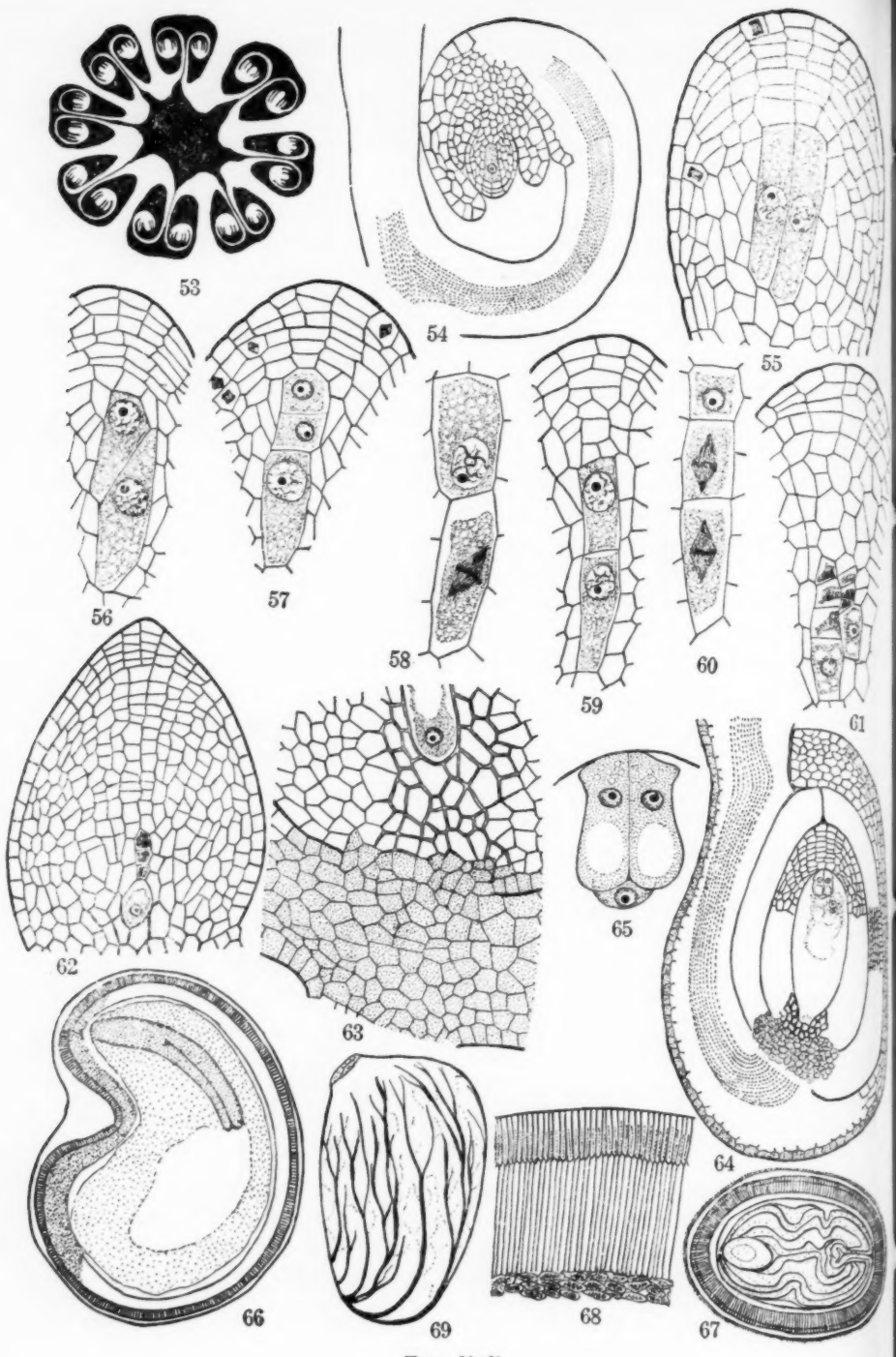
FIGS. 42–45. *Bombax malabaricum*. Fig. 42. Ovule primordium with multicellular archesporium, ×425. Fig. 43. Nucellus with full grown megaspore mother cell, ×425. Fig. 44. L.s fertilisable ovule, ×75. Fig. 45. Part of mature embryo-sac, ×200. FIGS. 46–52. *Eriodendron anfractuosum*. Fig. 46. Nucellus showing linear tetrad, ×425. Fig. 47. Fertilisable

ovule, ×90. Fig. 48. Thick-walled and tannin bearing cells subtending the embryo-sac, ×425. Fig. 49. Egg apparatus, ×425. Fig. 50. L.s. developing seed, ×10. Fig. 51. Micropylar part of a young seed showing nucellus, globular embryo and endosperm cells, ×285. Fig. 52. Seed coats from the young seed, ×75.

in a collateral or superposed position. In *Ochroma lagopus*, a fully formed 8-nucleate embryo-sac and a 2-nucleate one were seen in one ovule (Fig. 82). From their position they seem to have developed from megaspores of different tetrads. The archesporium in *Adansonia* differs markedly from that of other species, in the origin of the supernumerary cells which seem to be derived in two ways. In cases in which they are collaterally placed, they develop from separate archesporial cells (Figs. 55, 56, 61). Sometimes, they occur in a superposed manner. These are seen in ovules which are relatively well developed and in which the lower sporogenous cells are already undergoing meiotic divisions (Figs. 57–60). The upper cells which can be seen in all stages of development seem to be derived from one or more of the parietal cells as in several members of Malvaceæ (Stenar, 1925; C. V. Rao, 1953 *a*). These 'accessory sporogenous cells' are in most cases crushed before or after forming tetrads while the lower 'definitive sporogenous cells' function further and give rise to the embryo-sacs. Two fully formed embryo-sacs, however, were never met with in an ovule.

By a periclinal division, the archesporial cell forms the primary parietal cell to the outside and the megaspore mother cell to the inside (Fig. 73). The primary parietal cell undergoes first a periclinal division and by about this time in *Pachira aquatica*, the cells of the nucellar epidermis also undergo a periclinal division initiating the formation of nucellar cap (Fig. 33). The full grown megaspore mother cell has the usual elongated and tapering form and its nucleus stands near to its broad micropylar end (Fig. 75). There are several layers of nucellar cells below its chalazal end (Figs. 36, 37, 43). Due to the differences in the orientation of the spindle in the upper dyad cell in meiosis II, the megaspore tetrads have different forms linear (Figs. 38, 46, 77), T-shaped, or one in which the wall between the uppermost megaspores is inclined (Figs. 58, 76). Thirumalachar and Khan (1941) reported that the megaspore tetrads in *Eriodendron* are only T-shaped. In the present study however, T-shaped tetrads were found to be as common as the linear ones (Fig. 46). Their observation seems to be based on a study of a small number of ovules at this stage of development.

In all species investigated, the lowest megaspore functioned and gave rise to the 8-nucleate embryo-sac (Figs. 37, 61, 62, 78). The embryo-sac presents normal features. In general, the cells of the egg apparatus are almost of equal size (Figs. 49, 65). In *Ochroma*, however, the egg is much



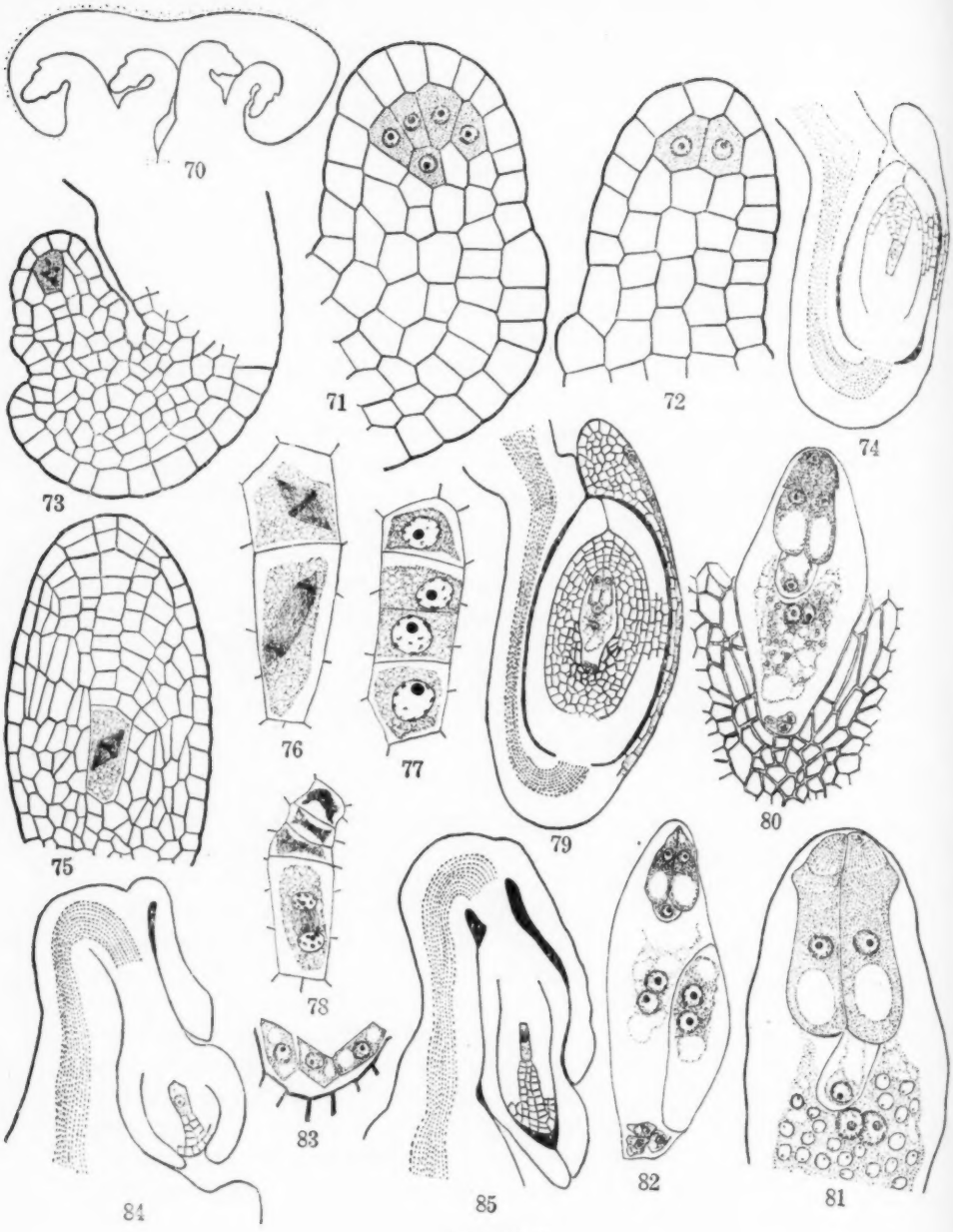
FIGS. 53–69

FIGS. 53–69. *Adansonia digitata.* Fig. 53. T.s. young ovary (diagrammatic; ovary wall not shown). Fig. 54. Young ovule showing megaspore mother cell, ×135. Figs. 55–61. Development of accessory sporogenous cells and tetrads, ×285. Fig. 62. Nucellus with single tetrad of which the lowest megaspore is enlarging, ×235. Fig. 63. Lower part of the ovule with 2-nucleate embryo-sac and the socket of thick-walled cells around it, ×285. Fig. 64. L.s. mature ovule, ×75. Fig. 65. Egg apparatus, ×425. FIG. 66. L.s. developing seed, ×4. Fig. 67. T.s. mature seed, ×3. Fig. 68. Part of the seed coats, ×30. Fig. 69. A seed of *Pachira aquatica* showing integumentary vascular bundles, ×1.

longer than the synergids which measure 70 and 50 μ respectively (Figs. 80, 81). The synergids are hooked. In *Ochroma* they show filiform apparatus (Fig. 81) in which respect they resemble cotton (Gore, 1932) and *Sida* (C. V. Rao, 1953 *a*). In *Pachira rosea* each synergid shows near to its micropylar end a rounded or oval patch with frilled rim which stains deeply with safranin (Fig. 41). As the pollen tube was always seen to penetrate the synergid through this patch, it seems to facilitate the entry of the pollen tube like the filiform apparatus. In *Ochroma*, the polar nuclei fuse just before fertilization while in other species they fuse much earlier (Figs. 45, 47). The antipodals are three in number, 1–nucleate and ephemeral except in *Ochroma* in which they last till the time of fertilization (Fig. 83). Persistent, supernumerary or multinucleate antipodals such as are found in the Malvaceæ were never encountered in Bombacaceæ. In *Pachira rosea*, *Bombax* and *Eriodendron*, the embryosac is larger (500–700 μ) and the cytoplasm appears relatively scanty (Figs. 40, 44, 47). In *Ochroma*, the sac measures about 120 μ in length and the cytoplasm appears relatively more abundant and is full of large starch grains which are not found in the embryo sacs of the other species (Figs. 80, 81).

FERTILIZATION

In *Ochroma lagopus*, the style is about 15 cm. long and the stigma is 5-lobed. The axial space of the ovary and the loculi terminate at the base of the style. In a young flower, 5 tracts of richly protoplasmic cells are seen in the style in line with the loculi. As the flower develops, these cells separate out schizogenously and form a space into which the peripheral cells project. These cells become elongated and finger-like and show extremely delicate cell walls and rich cytoplasmic contents; they collapse in fixed materials. These tracts extend up to the top of the style and seem to function as the transmitting tissue, feeding and directing the pollen tubes into the loculi. Near to the circumference of the style is seen a ring of mucilage canals some of which extend into the stigmatic lobes (Fig. 1). The remaining part of the style is made of the vascular bundles and uniform parenchyma. The style of *Ochroma* with separate tracts of transmitting tissue for the carpels, resembles that of the Malvaceæ.



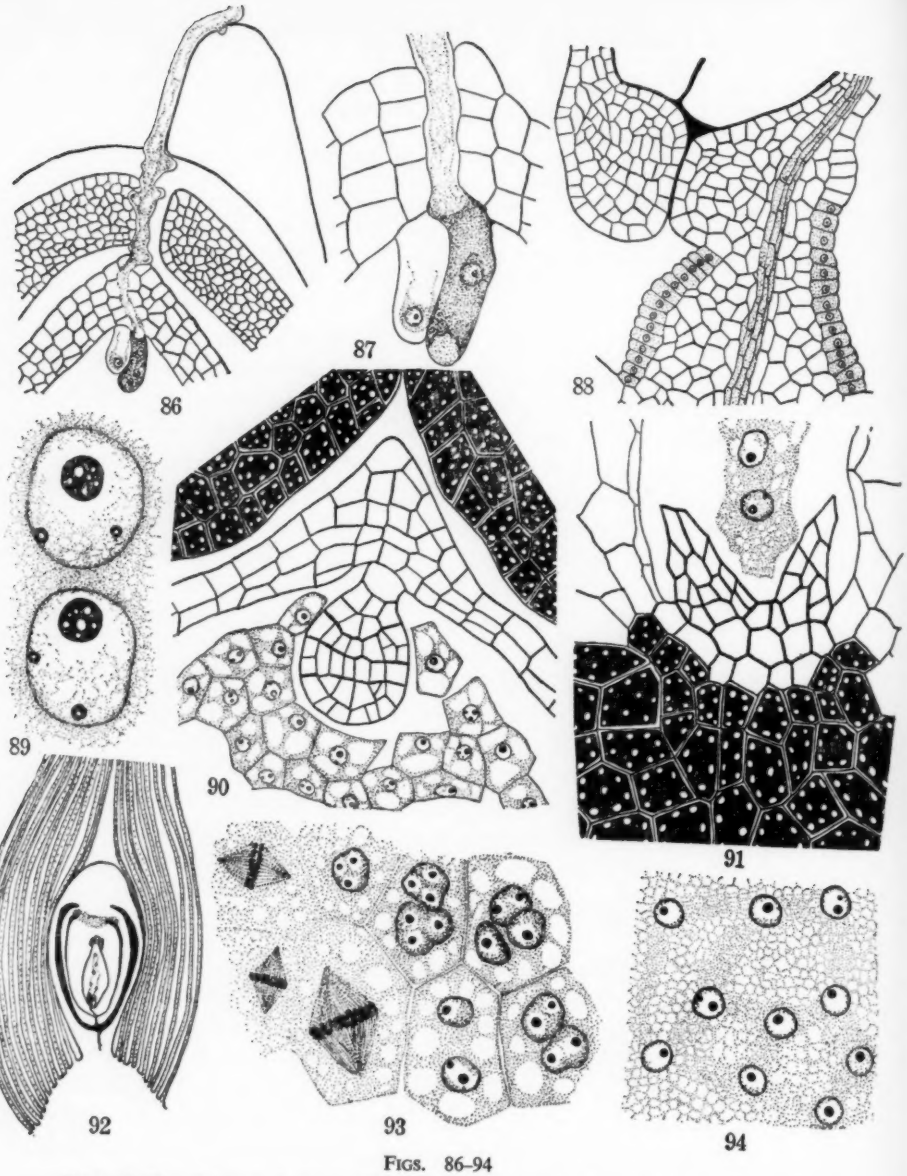
FIGS. 70–85

FIGS. 70–85. *Ochroma lagopus*. Fig. 70. T.s. loculus of the ovary showing ovule primordia, ×75. Fig. 71. Ovule primordium with multicellular archesporium, ×715. Fig. 72. Ovule with 2 functional archesporial cells, ×715. Fig. 73. Formation of primary parietal and megaspore mother cell, ×355. Fig. 74. Ovule with full grown megaspore mother cell, ×135. Fig. 75. First meiotic division in megaspore mother cell, × 355. Figs. 76 and 77. Tetrad formation, ×715. Fig. 78. Formation of 2-nucleate embryo-sac, ×425. Fig. 79. L.s. fertilisable ovule, ×115. Fig. 80. Embryo-sac with basal socket of thick-walled cells, ×285. Fig. 81. Part of the embryo-sac magnified; note filiform apparatus and starch grains in the sac, ×425. Fig. 82. Two embryo-sacs in one ovule, ×425. Fig. 83. Antipodals, ×500. Figs. 84 and 85. Malformed ovules, ×135.

The pollen tubes pass along the transmitting tissue, enter the loculi and curve near the bases of the funicles which are pretty long. The epidermal cells of the funicle are radially elongated and provided with dense cytoplasm and prominent nuclei (Fig. 88), and seem to nourish the pollen tubes which creep along their surface. Fertilization is porogamous. In the region of the micropyle the pollen tube which is about 10 μ in width gives off some short branches which end blindly (Figs. 86, 87), as in several Malvaceæ (Guignard, 1904; Stenar, 1925). The main tube carrying the gametic nuclei penetrates the 4–5 layered epidermal cap and enters a synergid through its micropylar end (Fig. 87). The affected synergid stains deeply thereafter and persists till the embryo becomes globular (Fig. 107). The pollen tube seems to exert some pressure on the basal wall of the synergid as Madge (1929) found in *Viola odorata*, because the synergid becomes elongated and cylindrical and equals the size of the egg (Fig. 87). Finally its wall at the tip gives way and the contents of the pollen tube are liberated into the sac. Early stages of fertilization were observed only in *Adansonia*; an embryo-sac at this stage showed an intact egg apparatus, a 2-nucleolate secondary endosperm nucleus at about the middle of the sac and higher up, two small spherical male nuclei each with a distinct nucleolus, and a degenerating tube nucleus.

Even after the discharge of the contents of the pollen tube, the synergid does not collapse in *Ochroma* but stiffly projects into the embryo-sac and even its nucleus persists for some time without showing any signs of degeneration (Fig. 87). The pollen tube persists and shows granular protoplasm like contents. As it is surrounded by the rich tannin and starch-bearing cells of the integuments in the micropylar region, the pollen tube seems to serve as a channel for the passage of nutritive materials as in several members of Malvaceæ (C. V. Rao, 1952). As the embryo develops, the starch grains disappear from the cells of the integuments and accumulate in the cells of the embryo from the 2-celled stage onwards (Fig. 97).

As in several Malvales, the fertilised egg undergoes a considerable period of rest while the primary endosperm nucleus divides soon after its formation.



FIGS. 86–94

FIGS. 86–92. *Ochroma lagopus*. Fig. 86. Upper part of the ovule showing branching pollen tube, ×200. Fig. 87. Part of the same magnified, ×425. Fig. 88. Funicle showing glandular epidermal cells, ×230. Fig. 89. Endosperm nuclei showing crystals in their nucleoli, ×715. Fig. 90. Micropylar part of a seed showing endosperm cells around the embryo and tannin and starch-bearing cells of the integument, ×285. Fig. 91. Postament and the subtending tannin and starch-bearing cells of the chalaza, ×285. Fig. 92. Fertilised ovule showing development of fibres, ×30. FIGS. 93 and 94. *Eriodendron anfractuosum*. Fig. 93. Cell formation in micropylar part of endosperm; note different sizes of spindles and polyploid nuclei and multinucleate endosperm cells, ×715. Fig. 94. Endosperm from the lower part of the embryo-sac, ×715.

During this period several noteworthy changes occur in the developing seed. It enlarges considerably, a seed with a 2-celled embryo in *Ochroma* measuring more than a millimeter in length as against 220 μ of the fertilisable ovule. The starch grains stored in the cytoplasm of the embryo-sac disappear completely. The embryo-sac expands from 120 μ to 800 μ. Its lower end extends to the chalaza and is invested by the socket of thick-walled cells which now projects into the sac as a postament (Figs. 91, 95). A similar structure was observed in *Tilia platyphyllos* (Stenar, 1925), and some members of Sterculiaceæ. By this time the cells in the chalaza enlarge considerably and store starch grains in abundance (Fig. 91). The cytoplasm of the sac extends to the postament and is hence in proximity to the cells of nucellus and chalaza containing reserve food materials. The integuments increase in thickness. Except the cells of the outer epidermis of the inner integument which is destined to develop into the palisade layer, all of their cells accumulate starch. The cells around the micropyle accumulate tannin also (Fig. 90).

Though the ovule in *Adansonia* is anatropous like that of other species studied, the developing seed becomes campylotropous unlike in others (Fig. 66), and resembles the seed of Malvaceæ. In *Eriodendron*, the seed grows uniformly for some time after fertilization so that it remains conical like the ovule. But later, it grows more in the lower part and the seed becomes almost spherical (Fig. 50). The embryo-sac expands enormously breaking down the basal socket of cells and becomes spherical; in the micropylar region, however, it is more constricted (Fig. 50).

Soon after fertilization in *Ochroma*, the glandular cells of the funicular epidermis grow out into long silky hairs (Fig. 92). Though these appear similar to the fibres in *Bombax* and *Eriodendron*, their origin is different. In the latter two genera, they represent epidermal outgrowths of the inner wall of the loculus; they form a packing material for the seeds which are themselves free from hairs. In *Ochroma*, on the other hand, they arise from the epidermal cells of the funicle which remains as a stout structure in the seed. So the seed is enveloped and dispersed by its own group of hairs.

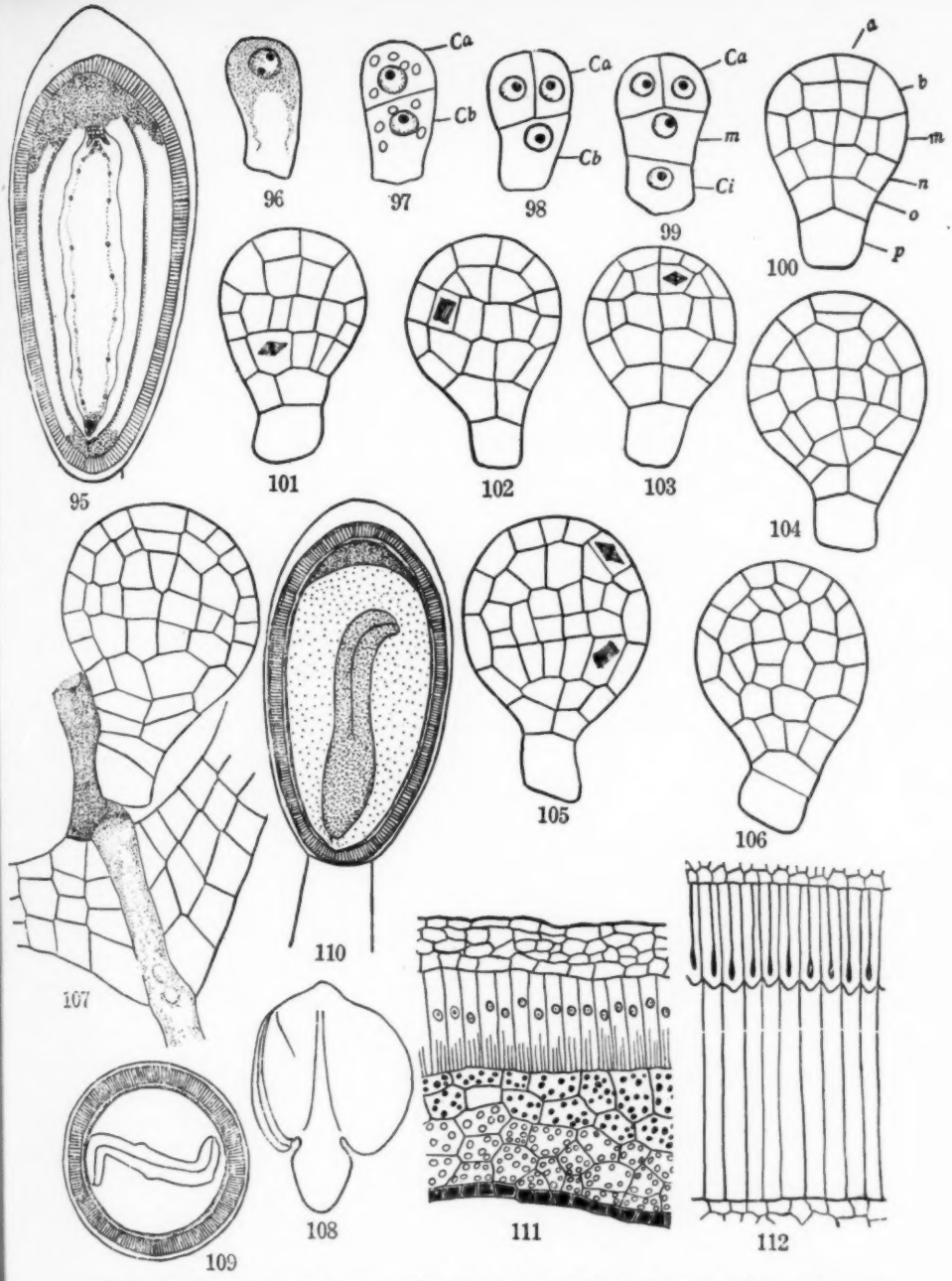
Endosperm

The endosperm is of the nuclear type. About 20 endosperm nuclei are formed by the time the fertilized egg divides. The cytoplasm of the embryo-sac forms a dense aggregation around the embryo and a strand extends to the postament. On the sides the cytoplasm is relatively thin and sparsely nucleate (Fig. 95). The endosperm nuclei are peculiar in that their nucleoli show 2–8 crystalline bodies which sparkle in refracted light (Fig. 89). There

are 1–3 nucleoli in each nucleus of which one is usually larger than the rest. Cell wall formation commences in the endosperm around the embryo when the latter has a globular form (Figs. 51, 90) by a process of furrowing and proceeds slowly towards the antipodal side. In *Eriodendron*, the endosperm nuclei in the constricted micropylar part of the embryo-sac are seen to be closely placed. These usually undergo fusions and give rise to lobed polyploid nuclei. They are larger in size, more densely chromatic and provided with more mucleoli than the endosperm nuclei lower down (Figs. 93, 94). The nucellar cells in the chalazal and micropylar regions remain intact for a longer time than those at the sides as in the seeds of Malvaceæ which also bears out that they are concerned in the nutrition of the developing embryo. The fully formed seed of *Ochroma lagopus* shows copious endosperm which surrounds a large straight embryo. The endosperm cells are densely packed with starch grains. The zone of tannin-bearing cells in the chalaza can still be seen as a dark mass of compressed cells (Fig. 110). The seed of *Pachira aquatica* is non-endospermic with a large fleshy embryo which shows foliaceous folded cotyledons.

EMBRYO

Development of the embryo conforms to the Urtica variation of the Asterad Type (Johansen, 1950), a feature which is characteristic of several Malvales (Figs. 96–107). The first division of the zygote is transverse and results in the basal cell *cb* and the terminal cell *ca* (Fig. 97). Usually the two cells undergo the next division simultaneously forming a T-shaped embryo of which the lower two cells are termed *m* and *ci* (Fig. 99). Occasionally, however, the division in the terminal cell which is longitudinal precedes that in the basal cell resulting in the formation of a 3-celled proembryo (Fig. 98). This gives definite evidence for the derivation of the median cell *m* from *cb*. Next, the two cells of the terminal tier divide longitudinally forming quadrants designated *q*. By this time *m* divides longitudinally and *ci* transversely to form two cells *n* and *n'*. The next division in *q* is characteristic: the cells divide by inclined walls resulting in an inner tier of rectangular cells *a* and an outer tier of triangular cells *b* (as seen in sectional view). The derivatives of this tier ultimately give rise to the cotyledons and stem tip. The two cells formed by *m* divide vertically and form circumaxially arranged quadrants; this tier by further divisions of the component cells forms the hypocotyl of the embryo. Divisions in *n* are similar to those in *m* and ultimately this tier develops into the root tip except its dermatogen and root cap. The cell *n'* divides transversely forming *o* and *p*. *o* behaves as the hypophysis and fills out the dermatogen of the root tip and the root cap and *p* functions as the suspensor initial. The demarcation of the dermatogen



FIGS. 95–112. *Ochroma lagopus*. Fig. 95. L.s. seed showing young embryo, micropylar and chalazal zones of tannin and starch-bearing cells, and postament, ×15. Figs. 96–107. Stages in the development of the embryo; note the persistent synergid and pollen tube in Fig. 107. All Figs. ×425. Fig. 108. Embryo, ×10. Figs. 109 and 110. T.s. and l.s. mature seed, ×10. Figs. 111 and 112. Seed coats, ×285.

initials occurs first in the tier *m* and then in *q* and *n* and later the common periblem-plerome initials undergo vertical divisions to demarcate the respective layers.

o divides twice vertically forming a plate of 4 cells. These undergo transverse divisions and form 2 layers of 4 cells each. The plate of 4 cells towards the embryonal mass forms the dermatogen of the root tip and that towards the suspensor functions as the root cap initiating layer. The suspensor remains 1-celled for a long time and is short in the mature embryo as in members of Sterculiaceæ.

The mature embryo is straight. The cotyledons are thick and fleshy and their margins are bent (Figs. 108–110). The cells of the embryo, like those of the endosperm, are rich in starch and other reserve food materials. As in *Pachira aquatica*, in *Adansonia digitata* also the whole of the endosperm is consumed in the mature seed; the embryo which is curved and provided with foliaceous cotyledons nearly fills the seed cavity (Fig. 67).

In *Ochroma*, some of the ovules abort after forming the 8-nucleate embryo-sacs. A few of the apparently well-developed seeds show only a cavity inside with a shrunken nucellus. The seed coats, however, are normally formed. Such exembryonate seeds which were also noticed in some Sterculiaceæ like *Sterculia colorata* and *Pterospermum heyneanum* by the writer, might have formed after the entry of the pollen tubes into ovules in which the embryo-sacs already commenced to degenerate. In *Adansonia digitata*, sterility is more common. All ovules in a developing fruit may degenerate. The fruit, however, develops normally and shows succulent wall which becomes very hard later. Seeds are very rare. This condition was found both in material sent by Dr. B. G. L. Swamy from Madras and by Mr. S. G. Krishna Murty from Vizianagram.

SEED COATS

In the seed of *Ochroma*, the outer integument becomes 5–6 layered and in *Eriodendron*, 7–8 layered. The outer 2–3 layers of cells in *Eriodendron* consist of tangentially flattened cells with deep staining tannin contents. The median layers consist of loose parenchymatous cells and the inner epidermis of more regularly placed cells constitutes the crystal layer. The seeds of *Pachira aquatica* are large and show integumentary vascular bundles (Fig. 69), in which respect they resemble the seeds of *Sterculia colorata* and *Pterospermum heyneanum* of Sterculiaceæ, and *Thespesia populnea* and *Gossypium* of Malvaceæ (Reeves, 1936). In *Adansonia*, the epidermal cells of the testa form 2–5 celled hairs by transverse divisions. Such hairs are not found in other genera studied, but are common in seeds of Malvaceæ.

In *Eriodendron* and *Ochroma*, the inner integument becomes 8–9 layered after fertilization. The outer epidermis as in all Malvales, develops into the palisade layer of radially elongated cells (Figs. 52, 111) which show a distinct light line (Fig. 112). This accounts for more than half of the thickness of the seed coats. Two or more layers of cells below the palisade layer form the outer pigment layer with deep staining contents. The next few layers of thin-walled parenchymatous cells are ultimately crushed. The inner epidermis or the ' fringe tissue ' consists of tangentially flattened cells whose cell walls stain deeply with safranin and whose lumens are filled with tannin.

Discussion

Some of the embryological features of Bombacaceæ are common to all the families of Malvales; there are others in which it resembles the members of the group of families Sterculiaceæ, Elæocarpaceæ and Tiliaceæ on the one hand and the Malvaceæ on the other. The features which are common for all the families of Malvales are: the accumulation of tannin and starch grains in the cells of the anther, connective, integuments and chalaza; shedding of the pollen grains in the 2-nucleate stage; crassinucellate bitegmic ovules in which the outer integument is less massive and grows faster than the inner; a zigzag micropyle; air space between the integuments; formation of an epidermal cap; *Normal-type* of embryo-sac development; porogamous fertilization; endotropic growth of pollen tube; nuclear endosperm which becomes cellular at a late stage in the development of the seed; development of embryo according to the Asterad Type (the only exception is *Muntingia calabura* (C. V. Rao, 1952 *a*) in which it keys out to the Onagrad Type); large embryo laden with food; absence of perisperm; details of development and structure of the seed coats, etc. The presence of so many common embryological features shows the homogeneity of the order.

The features in which Bombacaceæ resemble the members of the group of families Sterculiaceæ, Elæocarpaceæ and Tiliaceæ are: The secretory type of anther tapetum; marked secondary increase of the microspore mother cells; triporate smooth-walled pollen grains; anatropous ovules; organisation of a socket of thick-walled cells in the nucellus around the embryo-sac; the characteristic shape of the embryo-sac with broad micropylar and narrow chalazal ends; the formation of a postament in the seed; straight embryo-sac; hooked synergids; three 1-nucleate ephemeral antipodals; starch grains in the cytoplasm of the embryo-sac (*Ochroma*); a short suspensor for the embryo; straight embryo; massive endosperm and a chalazal outgrowth for the seed. There is particularly close resemblance between the

Bombacaceæ and Sterculiaceæ in the following features: The pollen grains of *Adansonia* and Dombeyeæ even in the details of sculpturing of the exine and structure of the germ pores; the pollen grains of *Pachira* and *Bombax* and Helicteræ; the synergids of *Pachira rosea*, and *Abroma augusta* and *Pentapetes phœnicea* (of Sterculiaceæ) in showing circular dark staining patches analogous to the filiform apparatus. In fact, a longitudinal section of the developing seed of *Ochroma* looks very much like that of *Abroma* and *Pentapetes*.

On the other hand, there are a few features in which Bombacaceæ resemble the Malvaceæ. Particular mention must be made of the development of some of the parietal cells of the ovule in *Adansonia* into accessory sporogenous cells; absence of starch grains from the embryo-sac (seen in five out of the six species studied); the fusion of the polar nuclei before fertilization; occurrence of wide branching persistent pollen tubes which seem to have a nutritive function; development of hairs in association with seeds which help in seed dispersal. The ovules of *Ochroma* resemble those of cotton particularly in being anatropous and showing synergids with filiform apparatus. The anatropous seed of *Pachira aquatica* with integumentary vascular bundles resembles that of *Thespesia populnea* and cotton. The campylotropous seed of *Adansonia digitata* with hairs developing from testa is closely similar to the type of seed commonly met with in Malvaceæ, *e.g.*, *Hibiscus micranthus*.

From the above account it is clear that embryologically Bombacaceæ differs from Malvaceæ as much as any other family of Malvales does and there is no justification for treating the family as a tribe of Malvaceæ as Bentham and Hooker (1862–83) did. On the other hand, a close comparison of the embryological diagrams of the different families shows that Bombacaceæ resembles more closely the group of families Sterculiaceæ, Elæocarpaceæ and Tiliaceæ than Malvaceæ. Bombacaceæ differs widely from Malvaceæ in the habit of the plants and the pollen grains. The resemblances between the two families seem to be due to their having evolved along parallel lines. Hutchinson (1926) arrived at a similar conclusion on the basis of morphological data (habit of plants). Setting aside the evidence of the unilocular anther, he split up the order into two the Tiliales and the Malvales, the latter comprising only the family Malvaceæ and the former the remaining families. But owing to the presence of the many common embryological, anatomical and morphological features among the various families of the order, Hutchinson's splitting of the order into two separate ones seems to be unwarranted. However, a division of the order into two sub-orders on

the lines suggested by Hutchinson could be advocated since it would indicate the affinities among the component families still retaining the fundamental integrity of the order.

Summary

Development and structure of the anther, pollen, ovule and embryo-sac were studied in the following members of Bombacaceæ: *Pachira aquatica* Aubl., *P. rosea*, *Bombax malabaricum* DC., *Eriodendron anfractuosum* DC., *Adansonia digitata* L. and *Ochroma lagopus* Sw. and fertilization and seed development in the last named species. Some observations were made on the seed structure of *Adansonia*, *Eriodendron* and *Pachira aquatica*.

The anther wall is 5–6 layered. The sub-epidermal layer develops into the fibrous endothecium and the innermost into the tapetum which is of the secretory type. The inner walls of the tapetal cells in *Ochroma* get cutinised and in *Pachira* they get gelatinised. Pollen tetrads are tetrahedral and cytokinesis is by furrowing. Pollen grains are 2-nucleate at the time of shedding. They are spherical in *Eriodendron*, *Adansonia* and *Ochroma* and triangular and oblately flattened in *Bombax* and *Pachira*. In general, they are smooth walled and triporate. In *Adansonia*, the exine shows knob-like outgrowths and ring-shaped harmomegathi around the germ pores.

The ovules are anatropous, bitegmic and crassinucellate and show a zigzag micropyle. There is a prominent nucellar cap and the cells in the chalazal region accumulate tannin and starch. A group of thick-walled cells is organised in the nucellus around the antipodal end of the embryo-sac; this later forms a postament. The primary archesporium of the ovule is multicellular to start with but usually only one cell functions. In *Adansonia*, some accessory functional cells develop from one or more parietal cells. Megaspore tetrads are both linear and T-shaped and the lowest megaspore forms the embryo-sac according to the *Normal*-type. The synergids in *Ochroma lagopus* show filiform apparatus and in *Pachira rosea* they show circular deep staining patches near to their micropylar ends through which the pollen tube penetrates. The polar nuclei fuse before fertilization and the antipodals degenerate by this time. Starch grains are found only in the embryo-sac of *Ochroma lagopus*.

The style in *Ochroma* shows 5 tracts of transmitting tissue of thin-walled richly protoplasmic cells. Fertilization is porogamous; the pollen tube gives off a few blind branches in the region of the micropyle and persists till the embryo becomes a globular mass and seems to serve as a channel for the transport of nutritive materials from the starch-bearing cells of the integuments surrounding it, to the embryo. The endosperm is nuclear and becomes

cellular by a process of furrowing. Embryo development conforms to the Urtica variation of the Asterad Type. The outer integument becomes 5–7 layered after fertilization and the inner 8–9 layered. The outer epidermis of the inner integument develops into the palisade layer with distinct light line. In *Ochroma*, hairs develop from the epidermal cells of the funicle and assist in seed dispersal. In *Adansonia* there are short woolly hairs on the testa. Mature seeds of *Ochroma* show copious endosperm and straight embryo. In *Adansonia*, the seeds are campylotropus and show a large embryo with folded foliaceous cotyledons. In *Pachira aquatica*, they are anatropous, non-endospermic and show integumentary vascular bundles.

The embryological features of Bombacaceæ show that the family is more closely related to the group of families Sterculiaceæ, Elæocarpaceæ and Tiliaceæ than to Malvaceæ. So there is no justification for treating the family as a tribe of Malvaceæ. On the basis of embryological data, a division of the order into two sub-orders on the lines suggested by Hutchinson is advocated, the first containing Sterculiaceæ, Elæocarpaceæ, Tiliaceæ and Bombacaceæ and the second the Malvaceæ.

ACKNOWLEDGEMENTS

The writer wishes to express his grateful thanks to Prof. A. C. Joshi and Prof. J. Venkateswarlu for their kind interest and helpful suggestions. His thanks are also due to Miss S. Yesoda Devi, Dr. B. G. L. Swamy, Mr. R. Seshagiri Rao and Mr. S. G. Krishna Murty for the materials of *Pachira* and *Adansonia* they kindly sent.

LITERATURE CITED

Banerji, I. .. "Development of the famale gametophyte and floss in ***Bombax malabaricum*** DC." *Proc. Indian Acad. Sci.*, 1942, **16**, B, 205–11.

Bentham, G. and Hooker, J. D. *Genera Plantarum*, London, 1862–83.

Brumfield, R. T. .. "Asymmetrical spindles in the first microspore division of certain angiosperms," *Amer. J. Bot.*, 1941, **28**, 713–22.

Engler, A. and Prantl, K. .. *Naturlichen Pflanzenfamilien*, Leipzig, 1895.

Gamble, J. S. .. *Flora of the Madras Presidency*, London, 1935.

Gore, U. R. .. "Development of the female gametophyte and embryo in cotton, *Amer. J. Bot.*, 1932, **10**, 795–807.

Guignard, L. .. "La double fecundation chez les Malvacees," *J. Bot.*, 1904, **18**, 296–308.

Hutchinson, J. .. *Families of Flowering Plants*, London, 1926.

Johanson, D. A. .. *Plant Embryology*, Waltham, Mass., 1950.

Iyengar, N. K. .. "Pollen tube studies in *Gossypium*," *J. Genet.*, 1938, **37**, 69–106.

Kosmath, L. .. "Studien über das Anthertapetum," *Osterr. bot. Zeitschr.*, 1927, **76**, 235–41.

Madge, M. .. "Spermatogenesis and fertilisation in cleistogamous flowers of *Viola odorata* var. *præcox*," *Ann. Bot.*, 1929, **43**, 545–77.

Rao, C. V. .. "Pollen grains of Sterculiaceæ," *J. Indian Bot. Soc.*, 1950, **29**, 130–37.

———— .. "Occurrence of persistent pollen tubes in Malvaceæ," *Curr. Sci.*, 1952, **21**, 49–50.

———— .. "The embryology of *Muntingia calabura* L.," *J. Indian Bot. Soc.*, 1952 *a*, **31**, 78–101.

———— .. "Floral anatomy and embryology of two species of *Elæocarpus*," *J. Indian Bot. Soc.*, 1953, **32**, 21–33.

———— .. "Embryological Studies [in Malvaceæ–I" *Proc. Nat. Inst. Sci. India*, 1954, **20**, 1953 *a*.

—— and Rao, K. V. S. .. "A contribution to the embryology of *Triumfetta rhomboidea* and *Corchorus acutangulus*," *J. Indian Bot. Soc.*, 1952, **31**, 56–68.

Rao, L. N. .. "Cytology of *Hibiscus trionum* L.," *New Phyt.*, 1941, **40**, 326–35.

Reeves, R. G. .. "Comparative anatomy of the seeds of cotton and other Malvaceous plants," *Amer. J. Bot.*, 1936, **23**, 394–405.

Stenar, H. .. "Embryologische studien. Zur embryologie der Columniferen," 1–75, *Akad. Abh. Uppsala*, 1925.

Thirumalachar, M. J. and Khan, B. A. "Megasporogenesis and endosperm formation in *Eriodendron anfractuosum* DC.," *Proc. Indian Acad. Sci.*, 1941, **14**, **B**, 461–65.

Willis, J. C. .. *A Dictionary of Flowering Plants and Ferns*, Cambridge, 1948.

ON THE LIFE-HISTORY AND BIONOMICS OF THE CARP MINNOW, *CHELA PHULO* (HAMILTON)*

By K. H. Alikunhi and H. Chaudhuri
(*Central Inland Fisheries Research Sub-Station, Cuttack*)

Received May 28, 1953
(Communicated by Dr. H. Srinivasa Rao, F.A.SC.)

Introduction

The genus *Chela* is widely distributed throughout India and as many as 10 species have been recorded from Indian waters by Day (1889) and a few more by subsequent workers. Of these *C. gora* and *C. untrahi* attain a length of 8 or 9 inches. The smaller species generally occur in large numbers, and yield valuable minor fisheries in certain areas. Most of them take baits and are therefore popular with anglers. In spite of their relative abundance in almost all inland waters, our knowledge of their bionomics and life-history is still very meagre. A very brief account of the food, feeding habits and development of *C. argentea* by Chacko *et al.* (1946), and a similar account of *C. untrahi* by Chacko (1951) appear to be the main contributions on these aspects at present. So far as we are aware, only passing references are made in other contributions on the bionomics of this genus.

Chela phulo is one of the smaller species of the genus and its distribution according to Day (*op. cit.*) is from Assam, through eastern and central India, to the Thungabhadra and Kistna basins. It is commonly found in ponds at Cuttack. This and other carp minnows generally abound in carp nurseries and stocking ponds. During an investigation of the causes of heavy mortality of carp fry in nursery ponds at Cuttack, attempts were made to elucidate the role of these 'weed' fishes in the economy of pond life. In the present paper the observations made on the breeding, fecundity, development, food and feeding habits of *C. phulo*, are detailed.

The fertilised eggs collected from the pond were identified as those of *C. phulo* by extensive comparison of the range and average size of the ovarian eggs of all the species of *Chela* commonly occurring in the locality, *viz.*, *C. phulo*, *C. bacaila* and *C. gora*. Besides, at the time of collection of the eggs, *C. phulo* predominated in the pond to the almost complete exclusion of other species of the genus. As the very few specimens of *C. bacaila*

* Published with the kind permission of the Chief Research Officer, Central Inland Fisheries Research Station, Barrackpore.

present in the pond were then immature, they could not have been breeding when *C. phulo* was spawning. The characters of the advanced post-larvæ rule out the possibility of any doubt in their identity.

Maturity and Breeding

Though reported to grow over 5 inches in length (Day, *op. cit.*) specimens longer than 3½ inches are rarely met with. The largest of over 600 specimens examined was only 85 mm. long. Sexual maturity is attained at a relatively small size; the smallest mature specimen, male or female, obtained being 54 mm. long.

Gonads were immature from October to January. By the middle of February some of the larger specimens (73 to 77 mm.) had the gonads in the IV-stage. By the end of March most of the males (54 to 67 mm.) had the testes in the V-stage. Females also then had the ovaries in the IV–V stage. Towards the last week of April some of the larger specimens (62 to 67 mm.) of either sex were found already spent. The majority of specimens were, however, fully ripe; while some small individuals, 48 to 50 mm. long had the gonads in the III stage (developing). Mature specimens were available till the middle of September. Though the breeding season thus extends from February to September, intensive breeding takes place only during April-May.

C. phulo breeds in stagnant ponds at Cuttack during the last week of May, when laid eggs were observed in the shallow margin of a small nursery pond, 50′ × 50′. The water was only 2½′ deep, and light greenish in colour, with a sparse bloom of algæ as the rains had not started. Spawning appears to take place usually in the morning (7-50 hours) as the fertilized eggs in their initial stages of cleavage collected on the 26th and 27th May, 1951 and on subsequent days indicate. The water was only a few inches (4 to 8″) deep at the marginal shallows where the eggs were found.

Sexual Dimorphism

Fresh mature specimens of both the sexes in the breeding season could be easily distinguished by the colour of the body and the fins as follows:

Distinguishing characters	Male	Female
(*a*) Ventral fins	Yellow	Whitish
(*b*) Anal fins	Yellow	Whitish
(*c*) Colour of body above lateral line	with distinct dirty yellow tinge	Dull; no yellow tinge
(*d*) Dorsal aspect of body ..	with a narrow dark streak medially	Entire dorsal aspect dark in colour.

There is no marked difference in size between the sexes. The largest male specimen in the present collection measured only 74 mm. long; while the few larger specimens obtained were all females, indicating that the females probably attain a slightly larger size than the males.

FECUNDITY

The fecundity of the species was studied by noting the total volume of the ovaries and then counting the number of ova in a sample piece of the ovary, the sample generally representing $\frac{1}{2}$ to $\frac{3}{4}$ of the total volume. The data gathered are summarised in Table I.

TABLE I

Data on the length, weight, maturity and facundity of Chela phulo *Hamilton*

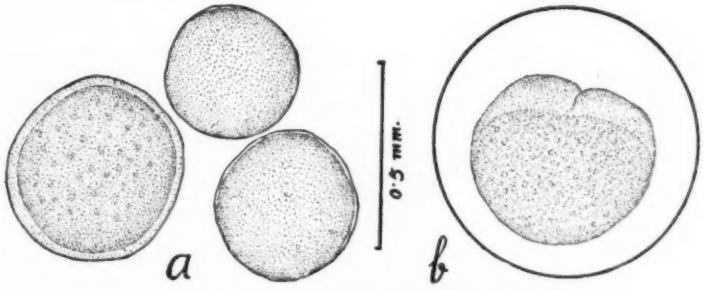
No. of fishes studied	Total length (mm.)		Aver. wt. (g.)	Stage of maturity	Vol.of ovaries (c.c.)		Total No. of ova		Aver. dia. of ovum (mm.)
	Range	Aver.			Range	Aver.	Range	Aver.	
4	57·0–59·0	58·25	..	V	0·12–0·20	0·15	1276–2512	1750	..
3	60·0–61·0	60·33	..	IV–V	0·10–0·20	0·16	1125–1660	1480	0·42
7	60·0–62·0	61·0	1·8	V	0·15–0·20	0·185	1296–2576	1912	0·465
6	63·0–66·0	63·7	1·92	V	0·13–0·20	0·157	1188–2092	1539	0·478
3	67·0–68·0	67·33	2·36	V	0·18–0·20	0·193	1679–2508	2026	0·483
3	73·0–77·0	75·0	3·35	IV–V	0·20–0·30	0·233	2092–2540	2309	0·446

It is seen from the table that generally fecundity increases with the size of the fish. Though the maximum number of eggs encountered in a single fish (62 mm. long and 1·9 gms. weight) is only 2576, when the size and weight of the fish are taken into consideration the fecundity is found to be exceedingly high. For instance, a specimen of the major carp *Cirrhina mrigala* weighing $3\frac{1}{4}$ lb. is reported to have as many as 2,16,000 eggs in the ovaries (Khan, 1947). On this basis, *i.e.*, when the weight of the fish and the number of eggs produced are compared, the fecundity of *Chela phulo* (weighing only about 3 gms. and producing over 2,000 eggs) is 4–6 times as high as that of *Cirrhina mrigala*. Though the ovarian egg shows a gradual increase in size with the size of the fish (Table I) the somewhat arbitrary estimation of the stage of maturity of the ovaries might well have contributed to render this increase quite distinct.

Development

The fertilised eggs collected from the pond were reared in the laboratory through the embryonic and larval stages, noting the details of development. Simultaneously the post-larval stages were also procured from the pond. These are described below.

Ovarian egg (Fig. 1 *a*).—The ripe ovary is pale or light grey in colour with the ova of different sizes. The fully ripe unfertilised ovum is nearly spherical, with a diameter of 0·41 mm. to 0·48 mm. The invisible vitelline membrane of the fresh egg becomes clear when properly dehydrated and cleared in clove oil.



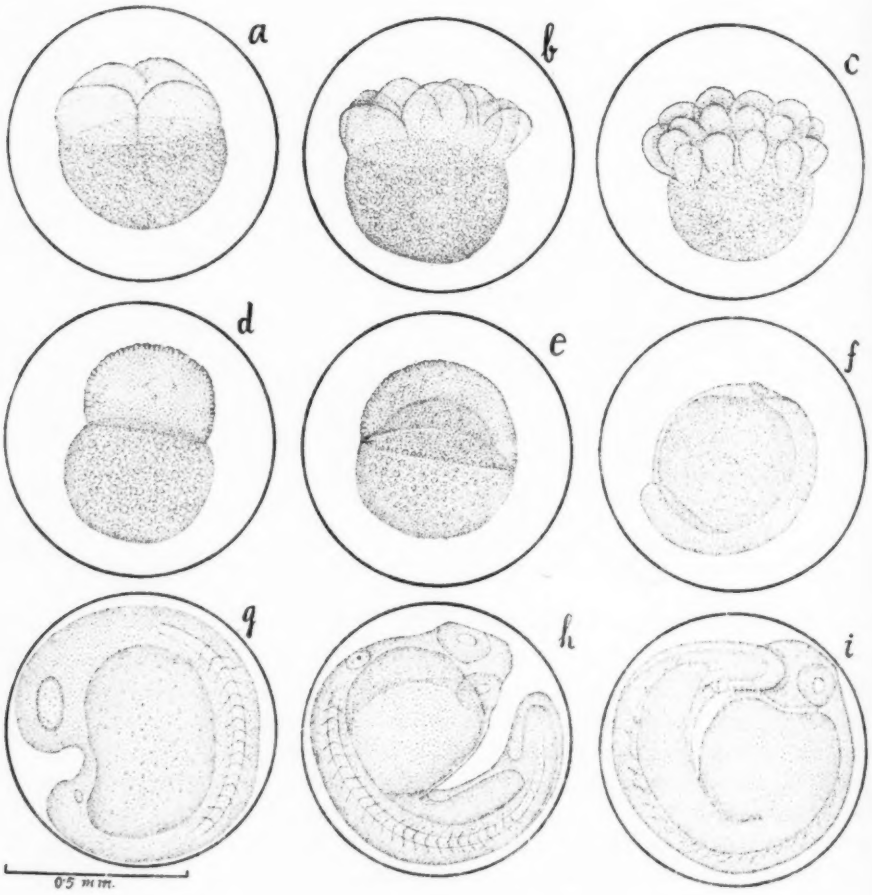
Text-Fig. 1. Development of *Chela phulo* Ham.
(*a*) Ovarian eggs, cleared in clove oil; (*b*) Fertilized egg undergoing first cleavage.

Embryonic development

Cleavage (Figs. 1 *b*; 2, *a-e*).—The small, demersal, somewhat sticky fertilised eggs settle at the bottom where particles of sand and debris stick to the egg membrane, but fall off when the egg is fixed in 5% formalin, leaving the egg membrane clear. After extrusion from the fish the egg swells rendering the vitelline membrane conspicuous with a fairly large perivitelline space between it and the egg (Fig. 1 *b*). The egg is now 0·675 mm. to 0·727 mm. in outer diameter, and 0·476 to 0·485 mm. in inner diameter which is slightly larger than the ovarian egg.

In the earliest embryonic stage collected from the pond which is not more than half an hour after fertilisation, the first cleavage has just commenced, and the blastodisc appears as a narrow, refractile area spread over one end of the yolk mass. The almost transparent egg contains coarsely granulated yolk. The first division divides the blastodisc into two large blastomeres. Five minutes later the second division takes place, followed by the 8-celled stage in another 7 minutes (Fig. 2 *a, b*). The fourth cleavage is effected

8 minutes later (Fig. 2 *c*), with the 32-cell stage following in the next 12 minutes. The morula stage is reached 1 hour after commencement of cleavage (Fig. 2 *d*).



TEXT-FIG. 2. Embryonic development of *Chela phulo* Ham.

(*a*) Fertilized egg, 4-celled stage; (*b*) Fertilized egg, 8-celled stage ; (*c*) Fertilized egg, 16-celled stage; (*d*) Fertilized egg, morula stage; (*e*) Fertilized egg, blastoderm cells spreading over yolk; (*f*) Fertilized egg, yolk invasion completed; embryonic rudiment laid; (*g*) Embryo, early C-shape; (*h*) Advanced embryo, about 8 hours after first cleavage; (i) Embryo ready for hatching; about 11 hours after first cleavage.

(All sketches by camera lucida, from fresh and freshly preserved material.)

Differentiation of the Embryo

2 *hours after first cleavage* (Fig. 2 *e*).—The crown of minute cells in the morula soon begins to spread over the yolk mass, about a third of which is covered within an hour after the morula stage.

4 *hours after first cleavage* (Fig. 2 *f*).—The blastoderm cells engulf the whole of the yolk mass; the embryonic rudiment is quite distinct though the head and tail portions are hardly distinguishable from one another.

5 *hours after first cleavage*.—The embryo is somewhat C-shaped, with the head, optic cups and the tail portions quite distinct, and 9 myotomes differentiated. The yolk is still a round mass.

6½ *hours after first cleavage* (Fig. 2 *g*).—Distinctly C-shaped, the embryo now fills the entire perivitelline space. The yolk mass has become oblong, about 0·48 mm. long. The number of myotomes has increased to 14 or 15. The optic cups are better differentiated and at the base of the projecting tail rudiment the Kupfer's vesicle has appeared as a small clear oval or oblong area.

7 *hours after first cleavage*.—The embryo shows occasional faint, twitching movements. The yolk mass has also assumed the characteristic shape, tapering towards the tail which is further elongated. The head is also more prominent. 20 myotomes have appeared.

7¼ *hours after first cleavage*.—The auditory cups have appeared. Both the head and the tail regions of the embryo are now moved frequently. The embryonic fin fold has also appeared around the tail. The posterior portion of the yolk is much drawn out and slender, though somewhat blunt.

8 *hours after first cleavage* (Fig. 2 *h*).—Two minute concretions appear in each of the auditory vesicles. More myotomes have appeared. The position of the anus is indicated very far backward, near the tail. Lens has appeared in the eye. The embryo is now able to change its position by frequent movement within the egg membrane.

8 *hours*, 40 *minutes after first cleavage*.—The heart begins to pulsate faintly. The embryo is very much elongated and is almost completely doubled up within the egg membrane.

11 *hours after first cleavage*.—The embryo is fully differentiated and almost ready for hatching, executing vigorous movements within the egg membrane.

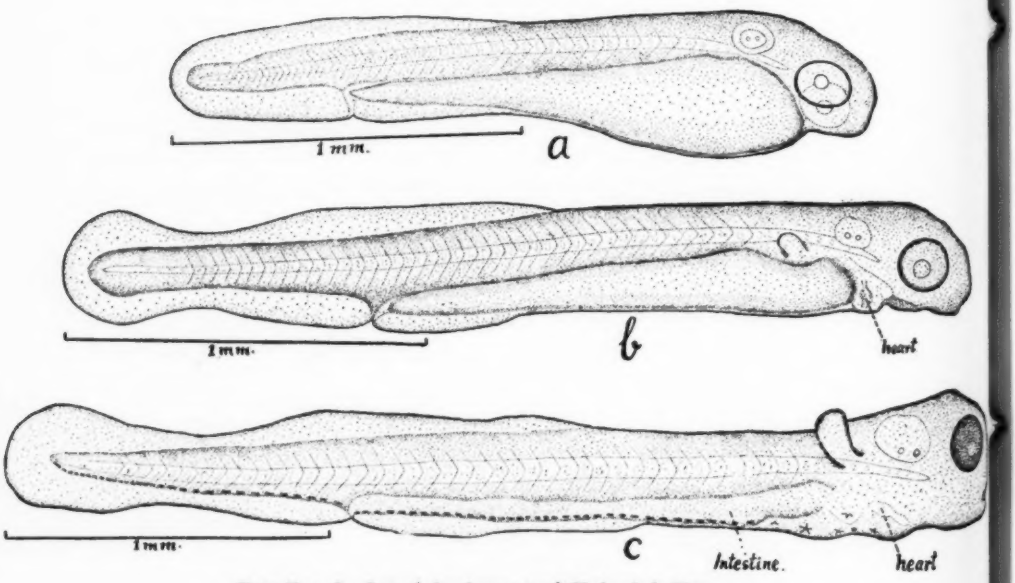
Within a few minutes all the eggs hatched out, almost simultaneously.

The cleavage and the entire embryonic development are completed in about 11 hours after the first division. The period of incubation, therefore, does not exceed 12 hours. The range of temperature of water in which the eggs were developing was 29·3 to 33·3° C.

Larval Development

Hatchling (Fig. 3 *a*)

Total length	— 2·025 mm.
Post-anal length of body	— 0·540 mm.
Length of yolk-sac	— 1·290 mm.
Maximum height of body	— 0·390 mm.
Maximum height of yolk-sac ..	— 0·285 mm.
Height of body at anal level ..	— 0·255 mm.



TEXT-FIG. 3. Larval development of *Chela phulo* Ham.

(*a*) Hatchling, 2·025 mm. long; (*b*) Larva, 12 hours after hatching, 2·565 mm. long; (*c*) Larva, 48 hours after hatching, 3·165 mm. long.

(Camera lucida sketches from freshly preserved material.)

The hatchling is very small and is almost transparent. The eyes are non-pigmented except for a faint dark tinge at the anterior margin of each. The heart, situated antero-dorsally to the yolk-sac, pulsates fairly rapidly. The auditory vesicles are more or less rounded. The concretions inside the

auditory vesicle are of almost equal size. Rudiment of the air bladder has appeared; while, the pectoral fin buds are absent. The yolk-sac is conspicuous. The embryonic fin fold is broad around the tail. Dorsally it extends forwards to a point almost midway between the tip of snout and the tip of tail. The narrow ventral fin fold in front of the anus extends anteriorly a little beyond the level of the dorsal fin fold. As the anus is situated far back the post-anal length of body is only just over $\frac{1}{4}$ the total length. 25 of the 42 myotomes are pre-anal in position.

Larva, 3½ *hours after hatching.*—

Total length	2·415 mm.
Length of post-anal region of body ..	0·675 mm.
Maximum height of body	0·375 mm.
Height of body at anal level ..	0·285 mm.
Length of yolk-sac	1·470 mm.
Maximum height of yolk-sac ..	0·255 mm.

The yolk-sac has become a little drawn out and appears much thinner. The pigment at the anterior margin of the eye is now distinct, with a small dark spot ventro-medially in each eye. The larva lies quiet at the bottom of the dish.

Larva, 12 *hours after hatching* (Fig. 3 *b*)

Total length	2·565 mm.
Length of post-anal region of body ..	0·835 mm.
Maximum height of body	0·345 mm.
Height of body at anal level ..	0·292 mm.
Length of yolk-sac	1·275 mm.
Maximum height of yolk sac.. ..	0·150 mm.

The eyes are pigmented, with their anterior portion darkest in colour, though the lenses still remain unpigmented. The buccal invagination has appeared. Rudiment of the air bladder is quite distinct. Auditory vesicles are not fully rounded in shape and the concretions inside are equal in size. Absorption of yolk is rapid and the yolk-sac now looks very thin and narrow. 26 of the 41–42 myotomes seen are pre-anal in position. The tip of the notochord is straight.

The larva generally lies quiet at the bottom of the dish but occasionally swims about.

Larva, 24 *hours after hatching.*—

Total length	2·955 mm.
Length of post-anal region of body	—1·005 mm.

The larva is extremely frail and slender. The mouth is now well formed though the lower jaw cannot yet be moved. The eyes have become dark. The pectoral rudiments have grown into larger flap-like functional structures enabling the larva to freely swim about in water. The yolk is almost completely absorbed except for a slender anterior portion. Along the mid-ventral line of the body the chromatophores are somewhat diffuse.

Larva, 36 hours after hatching.—Yolk is completely absorbed and the mouth has become fully terminal in position. The chromatophores have become slightly more distinct.

Larva, 48 hours after hatching

Total length	3·135–3·165 mm.
Length of post-anal region of body ..	1·005–1·065 mm.

The chromatophores along the ventral aspect form a continuous row from the pectoral region upto the notochordal tip. 36–38 of the 72 chromatophores are preanal in position. The larva is so slender and transparent that it is very difficult to find it even in a small beaker of water. It swims near the surface of water in a characteristic manner. The pectoral fins have further grown. The mouth is fully functional, though the gut was empty. In the auditory vesicle the posterior concretion has become distinctly bigger than the anterior. The embryonic fin fold is continuous and the tip of the notochord is not yet perceptibly upturned.

Larva, 72 hours after hatching.—There has been no appreciable growth or differentiation of structures after the 48-hour stage and the larvæ are not active. However, at the hind portion of the head 8–9 chromatophores have appeared on either side which merge with the ventral row of chromatophores. Mouth is larger than in the 48-hour stage.

Heavy mortality of larvæ started after the 48-hour stage. As there was no yolk at this stage the larva, under natural conditions, would have begun feeding. The excessive mortality in the laboratory might be due to lack of suitable food.

Post-larva, 4·815 mm. long (Fig. 4 *a*).—(Collected from Killa nursery pond No. 29, Cuttack, on 2–6–1951).

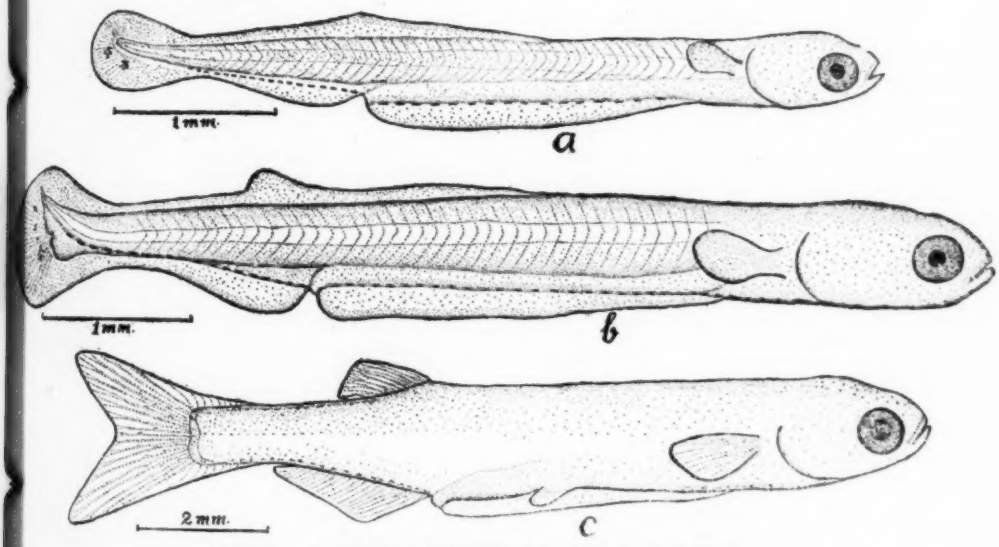
Length of post-anal region of body .. 1·545 mm.

Mouth is terminal and somewhat upturned. The embryonic fin fold is still continuous, with a slight broadening of the dorsal fin fold at about the level of the anus, indicating the position of the dorsal fin. The caudal is rounded. The tip of the notochord is slightly upturned. The ventral fin fold behind the anus is also somewhat broadened indicating the origin

of the anal fin. The number of chromatophores along the mid-ventral line is much less than in the 48-hour old larva, there being only 16 pre-anal and 17–18 post-anal chromatophores. This apparent reduction in number which occurs only in some larvæ is due to the merging of adjacent chromatophores which therefore appear larger and more conspicuous than in the larva. The young fish has obviously begun feeding as rotifers and rotifer eggs in the gut indicate.

Post-larva, 5·890 mm. long (Fig. 4 *b*).—(Collected from Killa nursery No. 29, Cuttack, on 2–6–1951).

Length of post-anal region of body .. 1·957 mm.



TEXT-FIG. 4. Post-larval development of *Chela phulo* Ham.
(*a*) Post-larva, 4·815 mm. long; (*b*) Post-larva, 5·890 mm. long; (*c*) Post-larva, 13·0 mm.. (All collected from pond and sketched immediately after preservation.)

The shape of the mouth resembles that of the adult. The dorsal and anal fin rudiments are more distinct but without fin rays in either. The rounded caudal fin is better differentiated though still continuous with the embryonic fin fold. Tip of the notochord is distinctly upturned and 12 rudimentary rays are present in the caudal fin. There are only 32 ventral chromatophores in a continuous line as those anterior to the anus have not yet merged into their adjacent ones. The 5–6 chromatophores on the caudal fin are more distinct than in the previous stage.

Post-larva, 13·0 *mm. long* (Fig. 4 *c*).—The dorsal, anal and caudal fins have been fully differentiated with the full complement of rays, 2/7–8, 2/19 and 20 respectively. The ventral embryonic fin fold persists in front of the anus. The bud-like rudiments of the ventral fins are distinct with 3–4 rudimentary rays. More chromatophores have appeared on the body, particularly on the dorsal aspect of the head and snout and also along the back from the level of the dorsal fin upto the caudal. The ventral row of chromatophores is now concentrated in the pectoral region behind the head and at the base of the anal fin upto the caudal.

Specimens 14·5 mm. long, have the ventral fin rudiments slightly better developed, with the ventral fin fold, persisting.

Post-larva, 16·0–17.0 *mm. long*.—Ventral fin fold has almost disappeared, though in some it is still seen as a very narrow strip. Ventral fins are fully differentiated with rays. Pectoral fins are elongated reaching halfway to the ventrals. The chromatophores are prominent at the nape, along the base of the anal fin, and behind the dorsal fin. The post-larva now bears considerable resemblance to the adult in all salient features.

FOOD AND FEEDING HABITS

Specimens of *Chela phulo* collected from nursery and stocking ponds in different months were examined to correlate the stomach contents with the items of food dominant in water at the time of collection. A detailed analysis of the gut contents of 158 specimens, 14·5 to 85·0 mm. in total length, made during the present study is briefly presented in Table II.

The number of items in the gut is relatively small. Cladocerans (*Moina* sp., *Pseudosida* spp.), copepods (*Diaptomus* spp., *Cyclops* spp. and *Nauplii*), Rotifers (*Brachionus pala*, etc.) insects (Chironomid and other dipteran larvæ) and the filamentous green alga, *Spirogyra* constituted the main items of food consumed. P'anktonic algæ (*Euglena*, *Closterium*, *Microcystis* and Naviculid diatoms) were seldom encountered in the gut and formed only a very insignificant portion of the feed. The proportion of vegetable debris was likewise very limited in the gut, which occasionally had some sand or mud particles also. It is fairly clear that the nature of the food taken depends to a large extent, on the item of food predominant in the environment at the time, but it is of interest to note that while the fish generally subsists on a predominantly zooplankton diet, will also feed on non-planktonic bottom living forms, like *Spirogyra*, aquatic insects, etc., in the absence or paucity of zooplankton. It has been observed that whenever zooplankton was rich in water the fish fed on it, the few algæ or insects taken with it perhaps being only

TABLE II

Chela phulo *Ham.*; *analysis of food consumed during different months in selected environments*

Date of collection	Time. Hours	Place	Nature of plankton	No. of fish examined	Range of total length (mm.)	Extent of feed (%)	Gut contents : Range and average % of items					Others
							Cladocera	Cope-poda	Rotifers	Insects	*Spirogyra*	
March 30-3-1950	15·00	Zobra. Cuttack, Pond No. 3	Zooplankton very poor	6	61·0–81·0	50–60	..	..	..	100·0	..	..
April 22-4-1952	8·30	Killa Channel, inlet section, Cuttack	Zooplankton poor; patches of algæ	15	60·0–74·0	30–80	0—40·0 (3·2)	0—3·0 (0·7)	..	0—25·0 (4·4)	50–100 (91·0)	0–3·0 (0·7)
April 22-4-1952	9·30	Do; section near nurseries	Algal growth less, zooplankton richer	16	48·0–57·0	40–80	0–100·0 (60·3)	0–20·0 (2·0)	..	0–100·0 (27·1)	0—40·0 (10·1)	0—9·0 (0·5)
April 26-4-1952	9·30	Killa, Cuttack, Pond No. 54	Zooplankton rich, sparse algal growth	12	58·0–83·0	60–100	30·0–100·0 (82·6)	..	..	0—20·0 (2·7)	0—70·0 (14·7)	..
May 26-5-1951	10·00	Killa, Cuttack, Pond No. 29	Zooplankton rich	14	14·5–40·0	50–100	40·0–100·0 (87·0)	0–60·0 (13·0)	..	..	..	..
				1	60·0	80	40·0	..	..	60·0	..	..
June 14-6-1952	9·30	Killa Channel, near nurseries, Cuttack	Do	40	19·0–68·0	60–100	97·0–100·0 (99·7)	0—1·0 (0·09)	..	0—2·0 (0·21)	..	..
September 11-9-1951	10·00	Do Pond No. 50	Do	4	64·5–78·0	70–100	80·0–100·0 (88·7)	0–15·0 (3·8)	..	0—20·0 (7·5)	..	..
December 12-12-1951	13·00	Do Pond No. 54	Do Rotifers	18	49·0–70·0	50–100	..	..	40·0–100·0 (77·0)	0—45·0 (16·5)	..	0–25·0 (6·5)
December 12-12-1951	12·00	Killa, Channel, near nurseries	Zooplankton poor	26	40·0–82·0	40–100	..	..	..	0·100·0 (77·4)	0–100·0 (22·6)	..
December 28-12-1951	11·00	Do	Do	6	51·5–72·0	70–100	..	..	0—3·0 (0·5)	0—15·0 (5·2)	80·0–100 (92·5)	0—5·0 (1·8)

UNIVERSITY OF MICHIGAN LIBRARIES

accidental inclusions, but when the planknton was poor there was a much larger proportion of *Spirogyra* and/or insects in the gut. Juvenile specimens feed almost entirely on zooplankton.

C. phulo appears to be an active, continuous feeder, judging from the fact that specimens with empty stomach were very rarely found. Even when the collections were made in the morning hours the amount of feed ranged from 30% to 80% showing that feeding activity had commenced early. Though the somewhat upturned mouth indicates a plankton feeding habit, the gut contents reveal that the fish feed also on insect larvæ and filamentous algæ at or near the bottom. Early post-larvæ feed largely on rotifers.

DISCUSSION

Like the majority of carp minnows, *C. phulo* breeds in stagnant waters, with a peak period of breeding just before the monsoon rains. The allied species, *C. argentea* and *C. untrahi* are reported to breed in running waters during the monsoons (Chacko *et al.*, *op. cit.*). The sexual dimorphism in the species, referred to above, occurs in the breeding season in *C. argentea* and *C. untrahi* also, the female exhibiting a patch of yellow or orange on the ventral portion of the body (Chacko *et al.*). Such pigmentation on the body proper is absent in *C. phulo* in which the males are more brightly coloured than the females. The ovarian eggs of *C. phulo* are less than 0·5 mm. in diameter, comparable in size to those of the larger species *C. gora* (0.65 mm.) and *C. bacaila* (0·6 mm.) (Alikunhi and Chaudhuri, unpublished). The ripe ovarian eggs of *C. untrahi* (diameter 0·75 mm. to 0·95 mm., Chacko, *op. cit.*) and of *C. argentea* (1·20 mm. to 1·50 mm. in diameter—Chacko *et al.*, *op. cit.*) are much bigger than the above. The size of eggs of *C. argentea* is unusually large for such a small-size fish and is almost as big as that of the ovarian eggs of the largest Indian carp, *Catla catla*. In the light of our observations on the eggs and fecundity of *C. phulo*, *C. bacaila* and *C. gora*, and in view of the similarity in the habits of the different species of the genus, this extraordinary size of the eggs in *C. untrahi* and *C. argentea*, if proved constant and a correct observation, would have to be correlated with the specialised environmental conditions, if any.

The fully swollen fertilised egg in *C. phulo* has a much less spacious perivitelline area than in the major Indian carps. No details of the embryonic and larval development of *C. argentea* and *C. untrahi* are available, but the cleavage and differentiation of the embryo observed in *C. phulo* show that the processes are rather rapid. The period of incubation in *C. phulo* is shorter by atleast 8 hours than in its congeners, *C. argentea* and *C. untrahi*. The hatchling is very small, without a mouth, gill slits or pectoral fins, and the

yolk is fully absorbed 1½ days after hatching. According to Chacko *et al.* (*op. cit.*) the hatchling of *C. argentea* has 4 gill slits at hatching, the mouth and eyes well formed, the yolk fully absorbed and is 8 mm. long in the next 4 hours. Similar conditions seem to prevail in *C. untrahi* also (Chacko, *op. cit.*). As compared to *C. phulo*, the rate of differentiation of structures in the above allied species is unusually rapid. The somewhat longer period of incubation of the egg (20 hours) in these latter may lead to a more advanced stage of differentiation of the embryo at hatching, but even so, it is doubtful whether the yolk would be completely absorbed within 4 hours after hatching.

C. phulo subsists largely on zooplankton, though often feeds on filamentous algæ and aquatic insects if zooplankton is scarce, but not on planktonic algæ as *C. argentea* does, in which the preference is appreciably less for micro-crustaceans (Cladocerans and Copepods). These comparisons can only give a very general picture of the food habits, when it is seen that the food consumed largely depends on the dominance of items of food present in a given environment.

As *C. phulo* draws largely on the zooplankton resources of the pond, it may be deemed an active competitor to the young fry of major carps which are almost entirely dependent on zooplankton (Alikunhi, 1952). The very careful eradication of this species from carp nurseries thus becomes a necessity to safeguard survival and growth of carp fry. In rearing tanks also the food habits of *C. phulo* will be incompatible, particularly with those of *Catla catla*, which, even after the early stages, continues to feed largely on zooplankton.

Summary

The common carp minnow, *Chela phulo* Ham., breeds in ponds from February to September. Intensive breeding takes place during May-June.

During the breeding season the mature males are easily distinguished from the females by their conspicuous yellow-tinged ventral and anal fins, and by other differences in pigmentation.

The fecundity of the species is relatively much higher than that of the major carps.

Eggs are generally laid in the morning, in the marginal shallows of ponds. The fully swollen fertilised egg is less than 0·75 mm. in diameter.

With the temperature of water ranging from 29·3 to 33·3° C., the early cleavages are completed and the morula stage reached within 1½ hours after fertilization, and the embryo hatches out within 12 hours.

The hatchling, just over 2 mm. long, is extremely frail, small and rather undeveloped, with the mouth and pectoral fins wanting. Absorption of yolk is completed 36 hours after hatching when probably the larva begins feeding. All except the ventral fins are differentiated when the post-larva is 13·0 mm. long. Ventrals are also fully formed in specimens 16·0 to 17·0 mm. long.

C. phulo is essentially a zooplankton feeder, but takes in appreciable quantities of filamentous algæ and aquatic insects also. No significant changes in the feeding habits have been observed in the different stages of its life. Food consumed, however, largely depends on the items of food available in the environment.

The feeding habits of *C. phulo* are incompatible with those of the early fry of the major Indian carps and of all stages of life-history of *Catla catla*.

ACKNOWLEDGEMENTS

We are indebted to Dr. H. Srinivasa Rao for the very valuable suggestions he has given us in the preparation of this paper. Our thanks are due to Dr. S. L. Hora for kindly checking up and confirming our identification of *Chela phulo*.

REFERENCES

Alikunhi, K. H. .. "On the food of young carp fry," *J. Zool. Soc. India*, 1952, **4** (1), 77-84.

——— and Chaudhuri, H. .. "On the role of 'weed' fishes in the economy of carp nurseries and stocking ponds" (Unpublished).

Chacko, P. I., Venkataram, R. S. and Ranganathan, V. "On the bionomics of the silver fish, *Chela argentea* Day," *Curr. Sci.*, 1946, **15**, 167.

Chacko, P. I. .. "Bionomics of the silver fish *Chela untrahi* (Day)," *Proc. 38th Ind. Sci. Congr.*, Calcutta, Part 3, Abstracts, p. 226,1951.

Day, F. .. *Fishes, Fauna of British India*, London, **1**, 1889.

Khan, M. Hamid .. "Development of fishes in the Punjab—III. Culture and propagation of indigenous species of fish," *Ind. Farm.*, 1947, **8** (9), 447–53.

196–54 Printed at The Bangalore Press, Bangalore City, by C. Vasudeva Rao, Superintendent and Published by The Indian Academy of Sciences, Bangalore

FISH MORTALITY IN THE ROCK POOLS OF A QUARRY NEAR VIRUDHUNAGAR (RAMANATHAPURAM DISTRICT), MADRAS

By P. I. Chacko and R. Srinivasan

(*Freshwater Fisheries Biological Station, Madras*

Received September 13, 1953

(Communicated by Dr. H. Srinivasa Rao)

1. Introduction

Sudden and large-scale mortality of fish often occurs in fish ponds causing considerable financial loss to the pisciculturist and reduction in the fish food resources of the country. Investigators have ascribed such mortality to physical, chemical and biological causes. Whipple *et al.* (1947) attributed the probable cause for the sudden death of a number of fishes (bass, roach, sunfish, suckers, eels, carps, etc.) in the lake at the Weequabic Reservation, U. S. A., to the complete exhaustion of oxygen resulting from the decay of algæ (*Clathrocystis* and *Anabæna*) which had been growing in the lake. In India similar phenomena have been recorded by Sewell (1927), Pruthi (1932) and Ganapati *et al.* (1950). Chacko and Ganapati (1949) have recorded an instance of large-scale mortality of fish in the Poondi Reservoir due to sudden depletion of water and the consequent choking of gills and gullets of fish with mud and filamentous algæ. Alikunhi *et al.* (1951) have found that heavy algal blooms in nurseries might sometimes lead to supersaturation of dissolved oxygen which may enter and accumulate in the stomach and subcutaneous spaces of the fishes and eventually kill them. But fish mortality in Madras State is due to asphyxiation brought about by oxygen depletion in the pond water, especially during nights or early hours of the day. The present paper describes briefly the investigations on fish mortality during the years 1950 to 1953 in the rock pools of a quarry at Virudhunagar which constitute a fish farm.

2. Description of the Virudhunagar Fish Farm

The utilisation of abandoned quarrys and mining pits for profitable fish culture is well known in America and Europe (Radcliffe, 1926 ; Edminster, 1947). On the outskirts of Virudhunagar Town, Madras State, are three quarry pools used by the Fisheries Department for rearing fish. These pits are big and deep enough to be perennial. Pool No. 1, locally known as

Pallivasal quarry pool, has a water-spread of about 1·3 acres. The Government-owned (known as *poromboke*) pools Nos. 2 and 3 are 0·3 and 0·4 acres in area respectively, with depths ranging from 6 to 18 feet.

TABLE I

Showing Particulars of Fish Stocked in the Quarry Fish Farm

Month of stocking	Kind of fish	Size in inches	Number stocked		
			Pool 1	Pool 2	Pool 3
May 1950	*Chanos chanos* ..	1–2	5,240	..	..
	Etroplus suratensis ..	1–3	..	25	..
June 1950	*Chanos chanos* ..	1–2	..	7,290	..
July 1950	*Etroplus suratensis* ..	1–2	..	540	..
August 1950 ..	Do. ..	1–2	14	..	200
	Gourami ..	12–13½	2	..	..
September 1950 ..	*Cyprinus carpio* ..	2–3	450	..	..
November 1950	*Labeo fimbriatus* ..	1½–3½	1,824	245	168
	Cirrhina cirrhosa ..	3–3½	848	210	..
December 1950 ..	Do. ..	3–3½	152	..	..
	Labeo fimbriatus ..	1½–3½	1,181	..	..
January 1951 ..	Do. ..	1½–3½	6,287	5,226	3,152
June 1951 ..	*Chanos chanos* ..	1–2	..	..	13,628
July 1951 ..	Do. ..	1–2	..	..	59
December 1951 ..	*Labeo fimbriatus* ..	1½–3½	..	..	850
May 1952 ..	*Chanos chanos* ..	1–2	..	2,500	..
June 1952 ..	Do. ..	1–2	..	7,290	..
October 1952 ..	*Labeo fimbriatus* ..	1½–3½	..	13	..
	Cirrhina cirrhosa ..	3–3½	..	85	..
November 1952 ..	*Labeo fimbriatus* ..	1–2	..	410	..
December 1952 ..	*Cirrhina cirrhosa* ..	3–4	..	3,386	2,881
January 1953 ..	Do. ..	3–4	..	920	825

The steep sides of the pools do not support vegetation. But macrophytes like *Chara zeylanica* were present in pool No. 1, and *Typha elephantina* occurred in the other two. The fishes present in the pools were *Barbus chrysopoma*, *B. melanostigma* and *Gambusia affinis*; but since 1950 *Chanos chanos*, *Etroplus suratensis*, *Labeo fimbriatus* and *Cirrhina cirrhosa* has been stocked as detailed in Table I. Trial nettings on 1st August 1951 showed that the growth of fishes in length and weight was as follows:

Kind of fish	Length in inches	Weight in lb.
Chanos chanos	16–20	0·50–1·00
Etroplus suratensis	4–6	0·25–0·38
Cyprinus carpio	15–20	2·00–3·00
Labeo fimbriatus	5–7	..
Cirrhina cirrhosa	5–7	..

This indicates that introduced species thrive and grow satisfactorily. But the total production of fish in 1950–51 in all the three pools was only 325 lb. due to loss of fish by periodical natural mortality.

TABLE II

Showing Hydrobiological Conditions of the Virudhunagar Quarry Fish Farm on 19th July 1952

Conditions	POOL NO. 1		POOL NO. 2		POOL NO. 3	
	Surface	Bottom	Surface	Bottom	Surface	Bottom
Time ..	11–30 a.m.	11–45 a.m.	10–30 a.m.	10·45 a.m.	11–0 a.m.	11–15 a.m.
Colour of water ..	Yellowish brown	Yellowish brown	Light green	Light green	Dark green	Green
Temperature, °C. ..	28·8	26·3	26·4	26·0	27·6	26·0
Turbidity, cm. ..	19·0	19·0	9·5	8·0	10·0	10·0
Dissolved Oxygen, c.c./litre ..	2·90	3·21	0·49	Nil	0·14	1·12
Do , % saturation	51·6	54·3	8·2	Nil	2·4	18·8
Free CO_2 p.p. 100,000 ..	0·50	0·60	Nil	Nil	Nil	Nil
Carbonates do ..	Nil	Nil	4·26	4·26	6·08	5·48
Bicarbonates do ..	21·64	28·45	63·08	66·79	17·32	17·32
Chlorides as Cl_2 do ..	65·0	65·0	22·0	19·0	47·0	47·0
pH ..	7·5	7·5	9·2	9·2	9·3	9·2
Cilicates as SiO_2 do ..	0·92	0·92	1·4	1·64	1·76	1·80
Nitrates as N do ..	Nil	Nil	Nil	Nil	Nil	Nil
Phosphates as P_2O_5 do ..	Trace	Trace	0·05	0·052	Trace	Trace
Macrophytic vegetation ..	*Chara Zeylanica*		*Typha elephantina*		*Typha elephantina*	
Invertebrate macrofauna ..	Water bugs, Snails, Crabs and Shrimps		Water bugs		Water bugs	
Plankton ..	Rotifers (*Brachionus*, *Noteus* and *Notholca*)—plenty *Microcystis*—few Copepods—rare Diatom (*Nitzschia*)—rare		*Microcystis* in bloom Rotifers (*Brachionus* and *Notholca*)—rare		*Microcystis* in bloom Daphnids—rare Copepods—rare Nauplius—rare Cyprids—rare	

3. FISH MORTALITY IN POOL NO. 1

On the morning of 5th July 1950 about 150 *Chanos chanos* (8 inches long) and a number of *Gambusia affinis* and *Barbus* spp. were found floating dead, and the mortality continued thereafter of a few fish daily for about a week. The hydrobiological conditions of the three pools on the 19th July 1950 were as detailed in Table II. Pool No. 1 differed from the others in (*a*) having higher content of dissolved oxygen, (*b*) lower pH, and (*c*) dominance of zooplankton. The higher oxygen content may have been due to the photosynthetic activity of the thick growth of the macrophytic vegetation. The low pH and the abundance of zooplankton were probably due to the death and decay of the excessive plant organisms. From 1st to 4th July it had been cloudy with slight drizzling on 4th evening, restricting photosynthetic activity on those days. The metabolic activities of the animal and plant communities in the pool must have also contributed to the exhaustion of almost all the dissolved oxygen in the water on the night of 4th July 1950, and to the suffocation and death of *Chanos chanos* during the early morning hours of 5th July 1950. The decay of plants may have also contributed to the early depletion of oxygen.

4. FISH MORTALITY IN POOL NO. 3

In this pool 220 *Chanos chanos* and 25 *Etroplus suratensis* were floating dead on 12th August 1950 followed by 22 more *Chanos* on the following day. The fishes were already in an advance stage of decay, indicating that mortality had taken place a few days earlier. Surface and bottom samples of water from this pool and in pool No. 1 where there was no fish mortality collected at different hours on the 14th and 15th August 1950 were examined for their hydrobiological conditions. The thermal and oxygen conditions of these pools are seen in Table IV. The differences between the two pools are tabulated below:

Pool No. 3	Pool No. 1
(*a*) There was a strong thermal stratification by day and an absence of 'turnover' by night as indicated by (i) a difference in temperature as high as 4·6° C. at 4 p.m. on 14th and 4·3° C. at 12 noon on the 15th between the surface and bottom layers throughout the day time; (iii) persistence of this difference even at 6 a.m. when homothermal conditions are usually observed; (iii) prevalence of almost	There was thermal stratification by day time, the maximum difference by day between the surface and bottom being 2·1° C. on 14th August and 3·7° C. on 15th August; these differences being much lower than those recorded for pool No. 3, in spite of the fact that pool No. 1 is deeper. There was a 'turnover' by night as indicated by the almost homothermal conditions prevailing from 10 p.m. of

Pool No. 3	Pool No. 1
uniform bottom temperature during day and night; and (iv) supersaturation of surface layer with oxygen, while the bottom layer continued to have a very low content of oxygen throughout the day and night.	August 14th to 6 a.m. of August 15th and by the uniform oxygen distribution during the early morning hours of 15th August.
(*b*) The oxygen content of the bottom layer was low and varied from 0·6 to 1·2 p.p.m.	The oxygen content of the bottom layer was high and was four to eight times the amount of oxygen contained in pool No. 3.
(*c*) There was an abundance of *Microcystis* and rotifers in the plankton. The photosynthetic activity of the blue-green alga *Microcystis* is responsible for the high oxygen content in the surface layers during day time. The respiratory activities of this alga and the rotifers which were next in the order of abundance, were also responsible for the low content of oxygen in the surface layers during the night.	The phytoplankton was poor; and rotifers were not so abundant as in pool No. 3. So, the amount of oxygen produced by day and consumed by night are also comparatively lower.

Thus the conditions were entirely different in the two pools. Where there was no turnover by night, fish mortality had taken place. It would appear that there had been no turnover for several nights prior to the mortality on 12th August 1950, resulting in the formation of a gradually increasing oxygen deficiency zone at the bottom especially during the night and of a very narrow upper zone with a moderate oxygen content, which was not sufficient to accommodate all the 8,000 and odd *Chanos chanos* and *Etroplus suratensis* stocked in the pool. Hence the mortality of a few hundreds of fish.

The third instance of fish mortality was also observed in pool No. 3. All the fish stocked were in distress, gasping for breath near the surface during the early hours of each day from 12th July 1952 onwards, and some of them even died as shown in Table III. As the death of fish continued throughout the period, July to January, mortality could not be attributed to high temperatures alone. In the course of the investigation of the cause of this mortality between 4th and 8th February 1953 a few *Cirrhina cirrhosa* also died, a post-mortem examination of which failed to give any satisfactory clue for their death. The thermal and oxygen conditions at different times of the day (Table IV) on the 5th and 6th February 1953 clearly show that

while there was a turnover by night in pool No. 2 there was none in pool No. 3. Further, the blue-green alga, *Microcystis*, was very abundant (as much as 13,550 individuals per ml. at 12 noon on 5–2–1953) in pool No. 3, whereas pool No. 2 supported only a moderate amount of this alga (about 3,125 per ml. at 12 noon on 5–2–1953). As already indicated, the presence of large number of organisms in pool No. 3 might have disturbed the biological balance in the pond, depleting on occasions almost all the contained oxygen, thereby causing the fish mortality. The rotting of the excessive algæ might have also hastened the depletion of oxygen.

TABLE III

Showing Weight of Dead Fish Removed from Pool No. 3

Date	Weight in lb.
13– 7–1952	7·00
15– 7–1952	8·50
17– 7–1952	4·75
20– 7–1952	4·25
22– 7–1952	7·50
24– 7–1952	8·00
27– 7–1952	6·00
29– 7–1952	80·00
30– 7–1952	23·00
31– 7–1952	30·00
3– 8–1952	8·50
30– 9–1952	4·50
2–10–1952	1·00
7–10–1952	4·00
17–10–1952	1·50
29–11–1952	1·00
1–12–1952	1·50
2–12–1952	2·50
3–12–1952	6·00
26– 1–1953	0·50

5. DISCUSSION

The three instances of fish mortality in the Virudhunagar quarry fish farm show how a disturbance in the biological balance of a pool, maintained by photosynthesis, respiration and bacterial decomposition, the three vital manifestations of the life processes in water, can lead to disastrous consequences. In pool No. 1 overgrown with the macrophyte, *Chara zeylancia*, and in pool No. 3 with the blue-green, *Microcystis*, in great abundance, respiration and decomposition made deep inroads on the oxygen content

TABLE IV

Showing the Thermal and Oxygen Conditions in the Virudhunagar Quarry Fish Farm

Date and time	POOL NO. 3 (12 ft. deep)						POOL NO. 1 (18 ft. deep)					
	Temperature, °C.			Dissolved oxygen, p.p.m.			Temperature, °C.			Dissolved oxygen, p.p.m.		
	Surface	Bottom	Difference	Surface	Bottom	Difference	Surface	Bottom	Difference	Surface	Bottom	Difference
14-8-1950—12 noon ..	31·2	27·4	3·8	9·7	0·6	9·1	30·0	28·3	1·7	6·4	3·4	3·0
4 p.m. ..	32·0	27·4	4·6	11·2	1·0	10·2	31·2	29·1	2·1	10·3	2·7	7·6
10 p.m. ..	29·8	27·4	2·4	10·5	0·7	9·8	29·2	28·8	0·4	9·3	4·1	5·2
15-8-1950— 2½a.m. ..	28·6	26·8	1·8	9·7	0·8	8·9	28·6	28·2	0·4	7·2	7·2	0·0
6 a.m. ..	28·0	26·6	1·4	5·3	0·8	4·5	28·2	27·8	0·4	6·0	5·9	0·1
							POOL NO. 2 (6 ft. deep)					
5-2-1953—12 noon ..	28·0	26·0	2·0	13·0	2·3	10·7	30·2	26·0	9·8	2·2	1·5	0·7
6 p.m. ..	26·3	25·0	1·3	8·4	0·5	7·9	26·3	24·2	2·1	4·7	0·0	4·7
6-2-1953—0·00 hrs. ..	24·2	23·1	1·1	7·7	0·7	7·0	24·0	24·0	0·0	1·0	0·1	0·9
6 a.m. ..	24·0	23·0	1·0	1·2	0·0	1·2	22·2	23·3	1·1	1·2	0·1	1·1
12 noon ..	29·8	26·3	3·5	2·9	0·5	2·4	26·2	26·2	0·0	0·3	0·3	0·0

of the water causing the death of fish already referred to. The oxygen produced by chlorophyll-bearing plants during day time is not always sufficient to meet the demands of aerobic and anærobic respiration. On a cloudy day when photosynthesis is limited, considerable oxygen deficiency may result overnight causing the death of fish as in pool No. 1. Kong (1952) has observed similar mortality amongst carps in Malaya on dull days. In pool No. 3 with an excessive growth of green plants, all the oxygen produced by day may be exhausted at night by respiration even during a bright season. On such occasions fishes generally die at night or during the early morning as observed by Hogan (1933). Powers (1937) thought that the sudden mortality of fishes was not brought about by asphyxiation but by a physiological derangement due to the alternate compensation of the blood to the higher and lower CO_2 tensions of the water at the bottom and surface respectively of the pond. But in our investigations free CO_2 was either completely absent or, when present, the difference in CO_2 tension between the surface and bottom layers was not so great (*vide* Table II) as to cause the death of fish. In the presence of a pronounced thermal stratification and absence of turnover in the pond, oxygen depression may increase the incidence of mortality. The plants at the surface extract dissolved substances from solution in the surface layers, which cannot receive much replenishment from below on account of the thermal stratification. Following exhaustion of this food supply, the plants decay reducing the oxygen at the surface even faster than it is absorbed by the air. Whipple *et al.* (1947) are of opinion that in shallow bodies of water the decay of algæ and organic matter may cause a depletion of oxygen sufficient to kill fish. Even though the decay of the algæ might not have been solely responsible for the death of fish investigated by us as indicated by the high oxygen values during morning hours, it would have certainly contributed towards a faster depletion of oxygen by night.

From the above it is evident that a heavy growth of algæ and macrophytes in a fish pond is not only undesirable, but definitely narmful if their growth is not kept under strict control. There are many methods in vogue to eradicate excessive aquatic vegetation. Whipple *et al.* (*loc. cit.*) have described several methods for the control of algæ. Srinivasan and Chacko (1952), who have reviewed them, found that Dicotox (an ethyl ester of 2, 4–D) was successful in killing weeds like *Hydrilla verticillata* and *Vallisneria spiralis*. The decaying algæ that float as a scum on the surface of the ponds should be removed periodically by dragging with a piece of cloth or net so as to prevent rapid exhaustion of oxygen by their bacterial decomposition.

6. Summary

Three instances of heavy fish mortality in the Virudhunagar quarry fish farm in Madras State are described. Oxygen depletion in the waters, especially during night and early morning hours, has been found to be responsible for the mortality in all of them. The oxygen deficiency in each case was traced to an excessive growth of green micro- and macro-vegetation which disturbed the biological balance, normally controlled by photosynthesis, respiration and bacterial decomposition. The deprivation of dissolved nutrients for the plants owing to the thermal stratification and absence of turnover in the fish farm was responsible for the death of the plants, the decay of which also contributed to a faster depletion of oxygen. The recurrence of such disasters to fish in farms can often be prevented by controlling the growth of micro- and macro-vegetation.

7. Acknowledgments

We are grateful to Dr. H. Srinivasa Rao for valuable suggestions and criticisms; and to the Director of Fisheries, Madras, for permission to publish this paper.

References

Alikunhi, K. H., Ramachandran, V. and Hiralal Chaudhuri — "Mortality of carp fry under supersaturation of dissolved oxygen in water," *Proc. Nat. Inst. Sci. India*, 1951, **17**, 261–64.

Chacko, P. I. and Ganapati, S. V. — "A case of large-scale mortality of fishes," *Sci. and Cul.*, 1949, **15**, 238–40.

Edminster, F. C. .. *Fish Ponds for the Farm*, Charles Scribners & Sons, New York and London.

Ganapati, S. V., Chacko, P. I. and Srinivasan, R. — "On a peculiar case of fish mortality in a section of the fort moat fish farm, Vellore," *Jour. Zool. Soc. India*, 1950, **2**, 97–100.

Hogan, J. .. "Experiments with commercial fertilisers in rearing large-mouth, black-bass fingerlings," *Trans. American Fish Soc.*, 1933, **63**, 110–19.

Powers, E. B. .. "Factors involved in the sudden mortality of fishes," *Ibid.*, 1937, **67**, 271–81.

Pruthi, H. S. .. "Investigations regarding a recent epidemic of fish mortality in the tank in the Indian Museum compound with remarks on the causation of such epidemics in general," *Revue. Hydrobiol. Hydrographic.*, 1932, **26**, 242–57.

Radcliffe, W. .. *Fishing from the Earliest Times*, 2nd Edition, London, 1926.

Swell, R. B. S. .. "On mortality of fishes," *Jour. Asiatic Soc. Bengal*, 1927, **22**, 177–204.

Kong, S. M. .. "The fitness of ecological niches into which fish are introduced at various stages, and the survival of the transplanted fish," *Proc. 3rd Meeting Indo-Pacific Fish Council*, 1951, 1952, **3**, 218–27.

Srinivasan, R. and Chacko, P. I. — "The control of aquatic vegetation with 2, 4-D," *Jour. Bombay Nat. Hist. Soc.*, 1952, **51**, 164–69.

Whipple, G. C., Fair, G. M. and Whipple, M. C. — *The Microscopy of Drinking Water*, 4th Edition, John Wiley & Sons, New York.

DIGESTION OF CELLULOSE BY THE MOUND BUILDING TERMITE, *TERMES* (*CYCLOTERMES*) *OBESUS* (RAMBUR)

By J. N. Misra and V. Ranganathan

(Technical Development Establishment, Laboratories, Kanpur)

Received July 18, 1953

(Communicated by Dr. T. S. Subramanian, F.A.Sc.)

1. Introduction

Among the few types of insects which can utilise cellulose as the main source of food (Trager, 1932; Ripper, 1930; Mansour and Mansour Bek, 1937) termites occupy an important position. The mechanism of digestion was little understood until Cleveland (1923, 1924) established the symbiotic relationship between certain orders of flagellates and lower termites which harbour a rich Protozoan fauna in the hind intestine. Working on the lower termites *Reticulitermes flavipes* and *Termopsis augusticollis*, Trager (1932) showed the presence of the enzymes cellulase and cellobiase in the gut of the insects. Unlike the lower termites, the higher termites (family—Termitidæ) which infest tropical or subtropical areas do not possess symbiotic flagellates. It was, therefore, considered of interest to investigate the origin, nature and properties of the enzymes present in the digestive tract of a higher termite *Termes* (*Cyclotermes*) *obesus* (Rambur) which was locally available.

2. Material and Methods

Insects

Termes (*Cyclotermes*) *obesus* (Rambur) is the common mound building termite found in the plains of India. This insect species maintains fungus gardens inside the mounds and has a well defined society consisting of various castes, *viz.*, king, queen, alates, soldiers, workers and nymphs. Since it is only the worker caste which causes damage to cellulosic materials, the emphasis in the present investigation has been on this caste. The insects used were collected locally (Kanpur).

Method of Preparing Gut Extract

Usually 800 to 1,200 guts were used in any one batch. The guts were placed in an agate mortar along with a small quantity of pure sand and ground into an uniform paste. This paste was diluted with distilled water

at the rate of 0·5 ml. for every 100 guts. On centrifuging (3,600 r.p.m.) for half an hour, the crude extract yielded a clear yellowish liquid. This was dialysed for 20 hours against running distilled water, after being covered with a layer of toluene to prevent bacterial contamination. The dialysed gut extract was used in the various experiments.

Estimation of Cellulase Activity

The cellulose suspension (0·4% w/v) was prepared according to the method of Fred and Waksman (1928) in which the filter paper dissolved in cuprammonium solution was reprecipitated by acid and subsequently washed free of acid. A known volume of a mixture of cellulose suspension and phosphate buffer of pH 4 was taken in a test-tube and to this was added an equal volume of the gut extract. The concentration of cellulose in the ultimate mixture was always 300 μ g./ml. The contents of the tube were covered with a layer of toluene and incubated at $35 \pm 1°$ C. for 24 hours unless otherwise stated and then the reducing groups were estimated.

Estimation of Cellobiase Activity

The cellobiose was prepared in the laboratory by the method described by Blatt (1943). A known volume of the cellobiose solution (0·06% w/v) made in phosphate buffer of pH 4 was taken in a test-tube and to this was added an equal volume of the gut extract so that the ultimate mixture contained 300 μg. of cellobiose/ml. The contents of the tube were covered with toluene and incubated at $35 \pm 1°$ C. for 24 hours unless otherwise stated and the reducing groups were estimated as described below.

Micro-Estimation of End Products

The end products of hydrolysis were estimated as reducing sugars, by the ferricyanide method of Hagedorn and Jensen (1923). In the case of cellobiose, a new calibration curve had to be drawn on the basis of estimations of known quantities of cellobiose. The degree of hydrolysis of cellobiose was calculated by difference from the curves for glucose and cellobiose.

3. Results

Presence of Cellulase and Cellobiase in the Gut Extract

The presence of the enzymes cellulase and cellobiase in the termite gut was established as follows:—To two tubes containing the gut extract were added separately cellulose suspension *plus* phosphate buffer of pH 5·6 and cellobiose solution in phosphate buffer of pH 5·6. The reducing groups

were estimated initially and after an interval of 4 hours. A considerable increase in reducing groups was observed in both cases, indicating that hydrolysis of cellulose and cellobiose had taken place. The degrees of hydrolysis of cellulose and cellobiose calculated from the increase in reducing groups were found to be 14·0 and 33·0% respectively. Additional evidence pointing to the existence of cellobiase activity was furnished by the identification of glucose (as the osazone m.p. 201–203° C., uncorrected) in the cellobiose hydrolysis.

Properties of Cellulase and Cellobiase

Optimum pH.—The optimum pH for both cellulase and cellobiase activities (as measured by per cent. hydrolysis of cellulose and cellobiose respectively) was estimated by using McIlvaine's Universal phosphate buffer. The results are represented in Fig. 1.

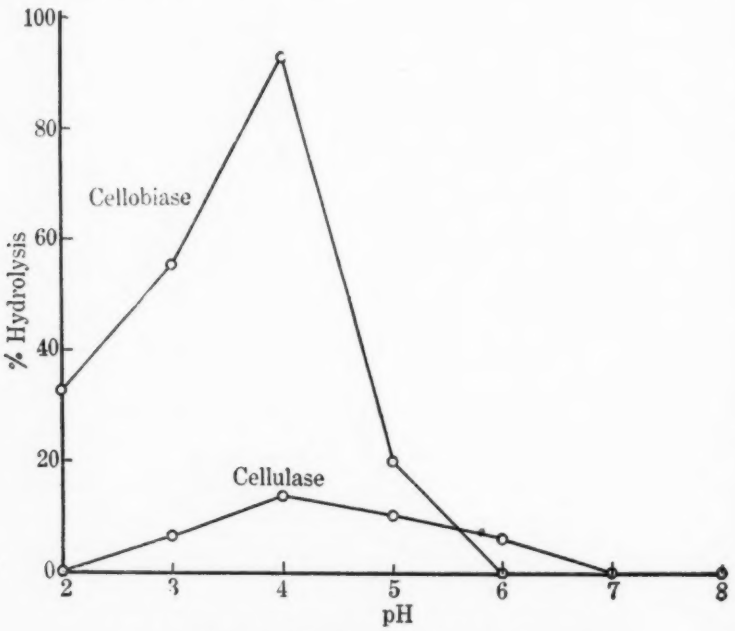


FIG. 1. Cellulase and Cellobiase Activities at various pH.

The results show that the optimum pH is about 4. In order to obtain a more accurate value, the activities at the pH range 3·4 to 4·8 were investigated. The results are represented in Fig. 2.

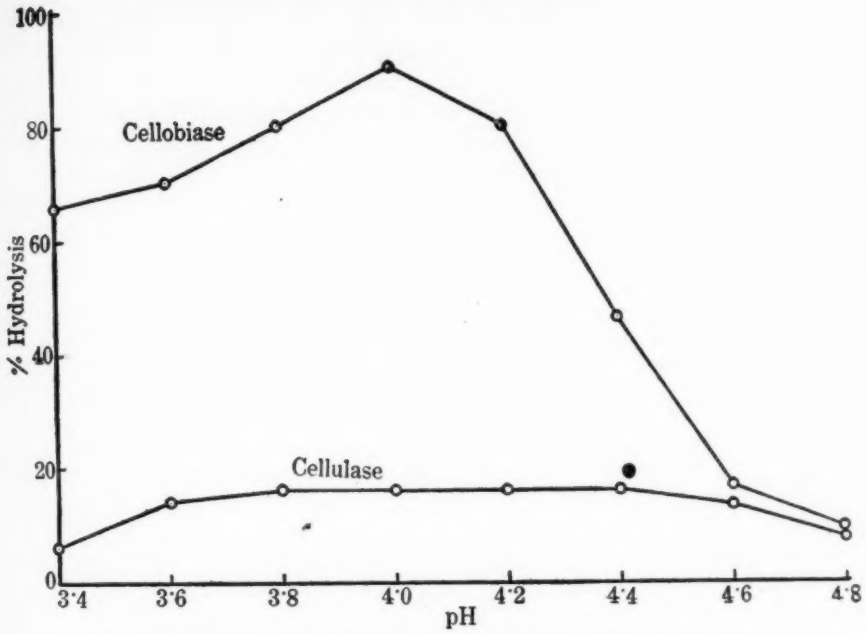


FIG. 2. Cellulase and Cellobiase Activities in a Restricted Range of pH.

Cellobiase has a sharp pH optimum at 4·0 while cellulase has an optimum pH range from 3·8 to 4·4. The values for optimum pH of cellulase from other sources vary from 4·5 to 5·5 (Greathouse, 1950).

Optimum Temperature.—In order to determine the optimum temperature for cellulase and cellobiase, test tubes containing the gut extract and cellulose suspension or cellobiose solution were incubated at different temperatures and the enzyme activities estimated. The results are represented in Fig. 3. The optimum temperatures for cellulase and cellobiase are 30° C. and 38° C. respectively. In the case of both these enzymes, activity ceases at about 50° C.

Heat Stability.—The heat stability of the enzymes was investigated by maintaining the gut extracts for one hour in water bath at different temperatures and then estimating the cellulase and cellobiase activities. The results are represented in Fig. 4. Both the enzymes are characterised by stability to heat and cellobiase is more so than cellulase, *e.g.*, cellulase hydrolyses only 8% of the substrate at 70° C., whereas with cellobiase the hydrolysis is 70%.

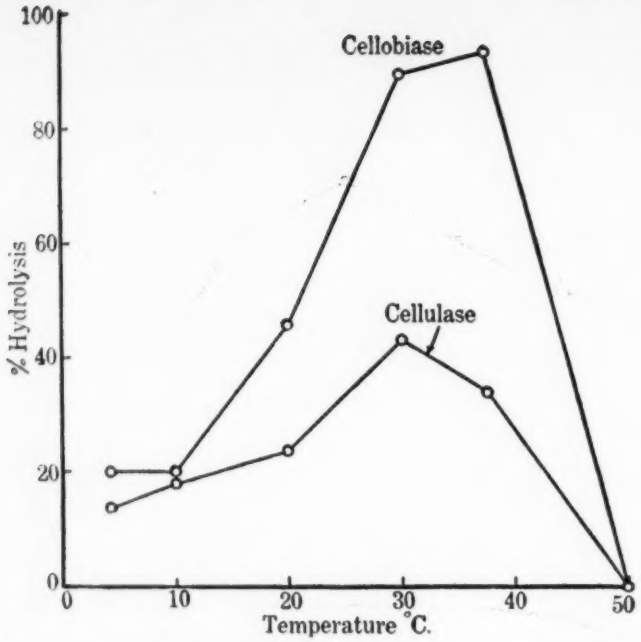


FIG. 3. Cellulase and Cellobiase Activities at Different Temperatures.

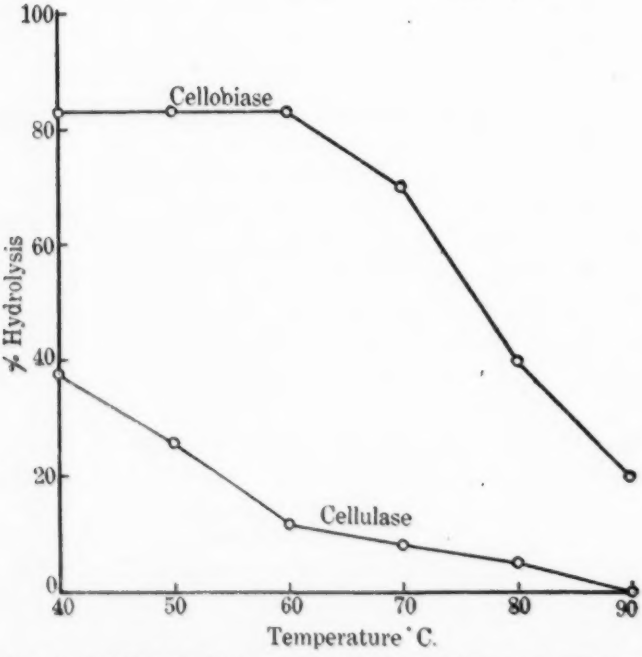


FIG. 4. Stability of Cellulase and Cellobiase at Different Temperatures

Cellulase and Cellobiase Activities and Period of Action

Cellulase and cellobiase activities were studied over a period of 72 hours, the extent of hydrolysis being estimated initially and at the end of 2, 4, 6, 8, 12, 20, 24, 48 and 72 hours. The results are represented in Fig. 5. In the case of cellulose, the extent of hydrolysis was only 32% in 72 hours, while with cellobiose, 73% hydrolysis resulted in 6 hours leading to its completion in 24 hours. The rate of hydrolysis with both the enzymes is more rapid in the initial stages.

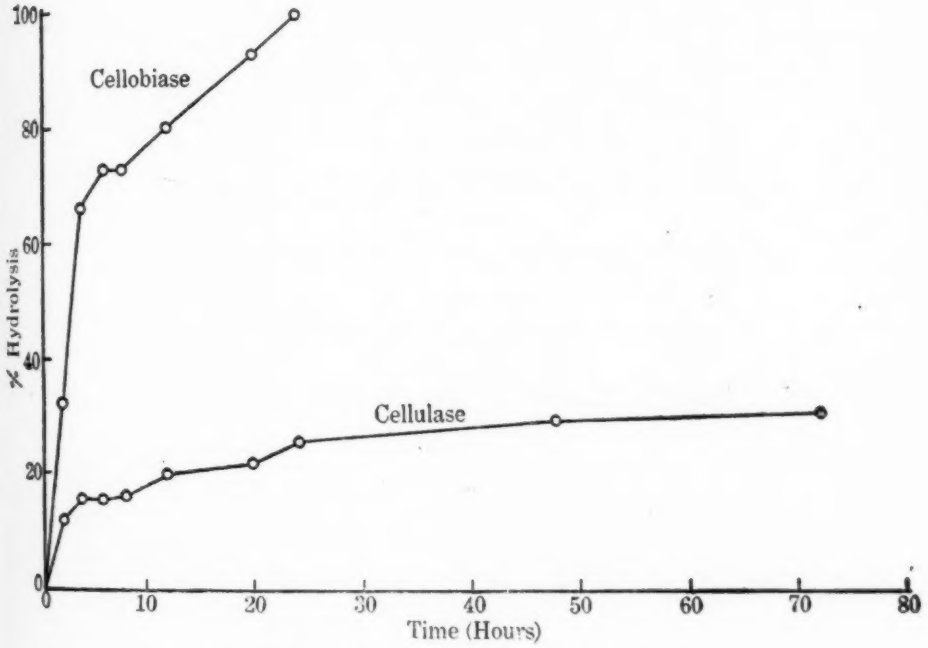


FIG. 5. Cellulase and Cellobiase Activities and Period of Action.

Cellulase and Cellobiase Activities in Relation to Concentration

To the gut extract, assumed to represent 100% concentration, water was added and a number of dilutions corresponding to 5, 10, 20, 30, 50 and 75 per cent. were obtained. Cellulase and cellobiase activities were estimated in respect of these dilutions. The results are represented in Fig. 6. It will be noted that the enzyme activity increases with concentration.

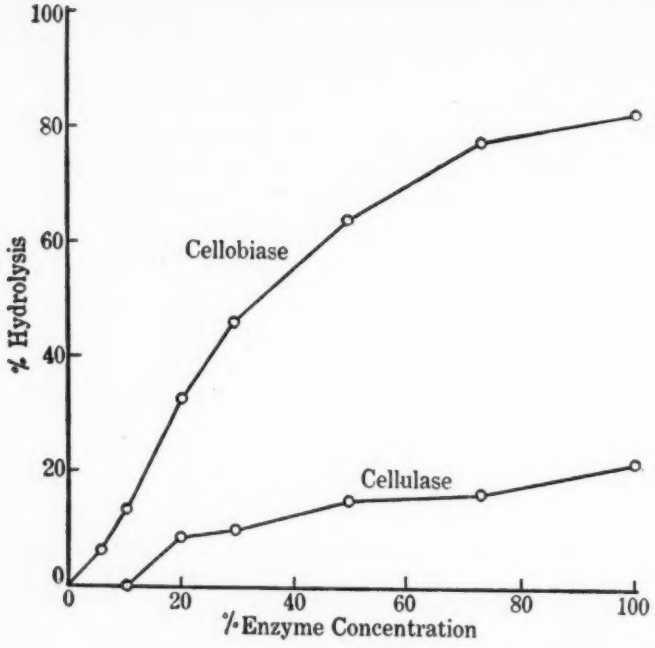


FIG. 6. Cellulase and Cellobiase Activities in Relation to Their Concentration.

Cellulase and Cellobiase Activities in Relation to Substrate Concentration

To the gut extract, varying amounts of substrate (cellulose or cellobiose) were added and cellulase and cellobiase activities estimated. The results are represented in Figs. 7 and 8. The quantity of substrate hydrolysed increases with the concentration of the substrate until optimum activity is recorded.

Cellulase and Cellobiase Activities of the Stored Gut Extract

The gut extract was maintained at 10° C. for varying periods. The activity was followed by estimating the extent of hydrolysis. The results are represented in Fig. 9. There was a gradual fall in activity after one week but after 3 weeks the activity tended to remain constant.

The Extra-Cellular Nature of Cellulase and Cellobiase

Cellulose destroying bacteria were isolated from the gut of termites according to the technique of Dickman (1931) and were cultured on

Omeliansky's medium. The activity of the gut extract after the addition of the mixed bacterial culture was investigated. The results are summarised

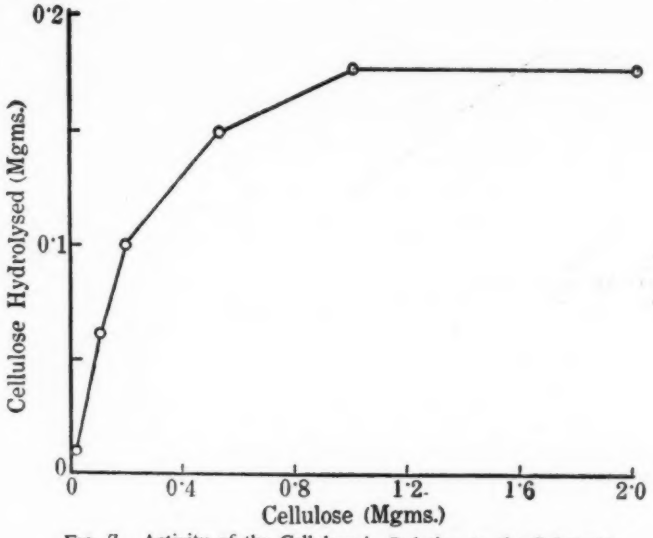


FIG. 7. Activity of the Cellulase in Relation to the Substrate.

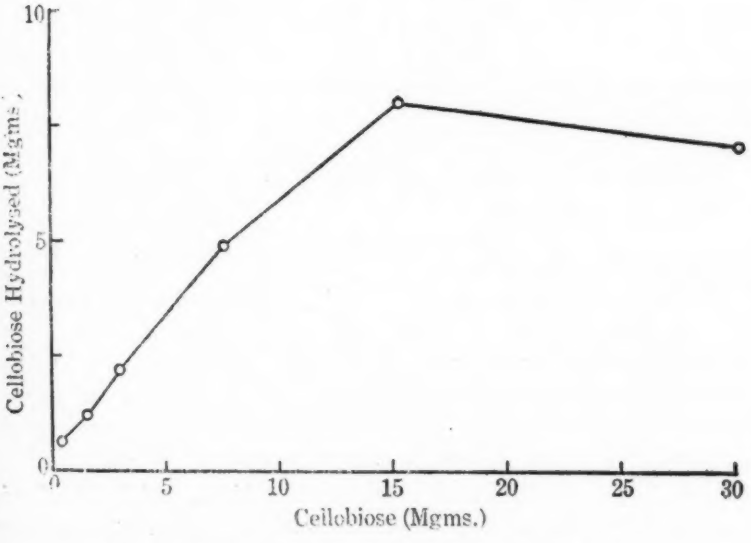


FIG. 8. Activity of the Cellobiase in Relation to the Substrate.

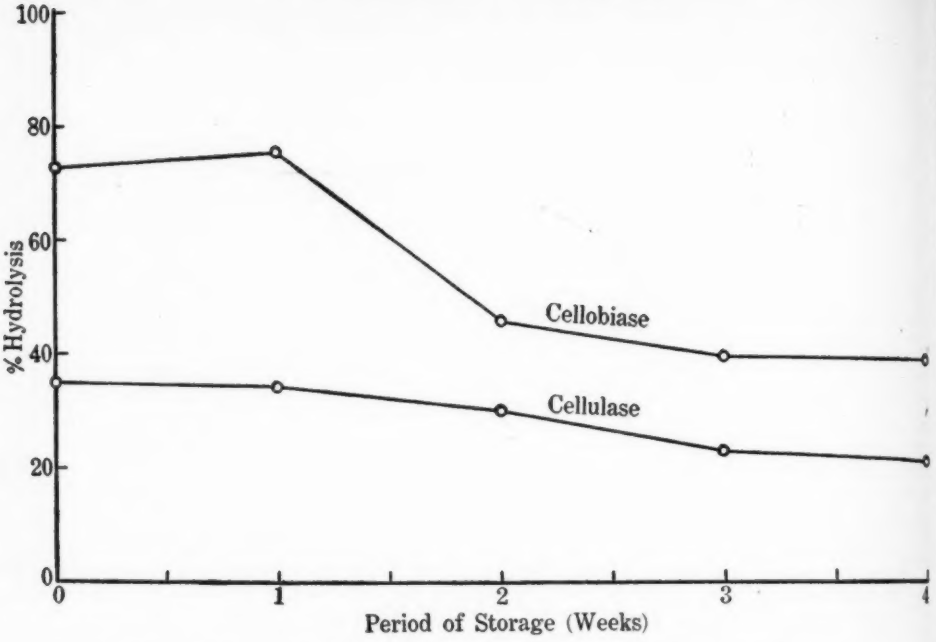


FIG. 9. Cellulase and Cellobiase Activities in Relation to the Storage of Gut Extract.

in Table I and they show that there is no increase in the activity of enzymes although bacteria had been added. This finding furnishes indirect evidence on the extra-cellular nature of the enzymes present in the gut extract.

TABLE I

Cellulase and Cellobiase Activities with the Addition of Bacteria

Description of experiment	Volume of toluene added in ml.	% Hydrolysis (at 35 ± 1° C.) at the end of 24 hours	
		Cellulose	Cellobiose
Bacteria added to gut extract ..	0·5	14·0	70·0
Gut extract alone (control) ..	0·5	23·0	74·0

Cellulase and Cellobiase Activities in Different Regions of the Gut

The extracts obtained from (i) the fore and midgut, and (ii) the hindgut were examined for cellulase and cellobiase activities. The results are shown in Table II.

TABLE II

Cellulase and Cellobiase Activities in Different Regions of Gut

Region from which extract was made	% Hydrolysis (at 35 ± 1° C.) at the end of 24 hours	
	Cellulose	Cellobiose
Fore and midgut	0	6
Hindgut	15	57

In the fore and midguts, slight cellobiase activity is present, but cellulase activity is completely absent. The hindgut shows considerable cellulase and cellobiase activities. It follows, therefore, that the digestion of cellulose takes place in the hindgut. A similar finding has been recorded by Trager (1932) working on lower termites.

Cellulase and Cellobiase Activities in Castes of Termites other than Workers

Cellulase and cellobiase activities in the gut extract of castes other than workers namely king, queen, alates (winged forms), soldiers and nymphs were estimated. The results are shown in Table III.

TABLE III

Cellulase and Cellobiase Activities in various Castes of Termites

Caste	% Hydrolysis (at 35 ± 1° C.) at the end of 24 hours	
	Cellulose	Cellobiose
King	0	14
Queen	0	14
Alates (winged form)	0	33
Soldiers	0	0
Nymphs	0	0

It will be observed that soldiers and nymphs are completely devoid of the two enzymes and that only slight cellobiase activity is shown by the king, queen and alates.

The Action of Cellulase on Certain Cellulose Derivatives

Three cellulose ethers obtained from Messrs. Imperial Chemical Industries (India) Ltd., were used as substrates for termite cellulase. The ethers were Cellofas TWF, Cellofas TAF (Sodium salts of carboxy methyl cellulose) and Cellofas TWL (methyl ethyl cellulose). Of these the first and the last one were soluble in water. 0·75 ml. of gut extract was taken and reacted with 0·45 mgm. of substrate in 0·75 ml. of phosphate buffer of pH 4. The percentage hydrolysis was calculated according to the method of Holden & Tracey (1950). The results are shown in Table IV.

TABLE IV

Action of Termite Cellulase on Cellulose Derivatives

Description of cellulose derivatives	Trade name	Degree of substitution	% Hydrolysis (at 35 ± 1° C.) at the end of 24 hours
Methyl ethyl cellulose ..	Cellofas TWL	$-OCH_3$ 0·4 $-OC_2H_5$ 0·9	7
Sodium salt of carboxy methyl cellulose	Cellofas TWF	0·45–0·5	36
Do. ..	Cellofas TAF	0·18	45

Inhibitors for Termite Enzymes

In a search for efficient inhibitors for the termite cellulase and cellobiase, zinc sulphate, chromium chloride and copper sulphate were investigated. The results given in Table V show that at 0·1 M concentration zinc and chromium inhibit cellulase activity almost completely. Zinc at 0·005 M concentration inhibits cellobiase activity slightly but a further increase in the concentration does not alter appreciably the extent of inhibition. Chromium lowers the cellobiase activity considerably at 0·1 M concentration.

4. DISCUSSION

Cellulose Digestion in Termites

Beeson (1941) has stated that the hindgut of a majority of wood-eating species of termites contains rich fauna and flora comprising mostly of

TABLE V

Cellulase and Cellobiase Activities in the Presence of Metallic Salts

Description of the salt	Molar concentration of the salt in the final mixture	% Hydrolysis (at 35 ± 1° C.) at the end of 24 hours	
		Cellulose	Cellobiose
$ZnSO_4$ $7H_2O$ ..	0·005 M	18	53
$CrCl_3$ $6H_2O$ ..	,,	10	60
$CuSO_4$ $5H_2O$ ..	,,	10	70
Control ..	..	16	56
$ZnSO_4$ $7H_2O$ ..	0·05 M	8	49
$Cr Cl_3$ $6H_2O$ ..	,,	5	43
$CuSO_4$ $5H_2O$ ..	,,	*	*
Control ..	..	22	86
$ZnSO_4$ $7H_2O$ ..	0·1 M	4	53
$CrCl_3$ $6H_2O$ ..	,,	0	20
$CuSO_4$ $5H_2O$ ..	,,	*	*
Control ..	..	22	73

* Estimations could not be carried out due to interference from the copper ions.

Protozoa, bacteria and fungi and that it is only Protozoa which contain enzymes that are able to hydrolyse cellulose. Those species of termites, belonging to family Termitidæ, for example, *Termes* (*Cyclotermes*) *obesus* (Rambur) which do not contain intestinal Protozoa do not usually feed on sound timber but obtain their food requirements from other sources like decayed timber, fungi, humus and organic matter derived from soil. The results reported in this paper, show that bacteria present in the gut of the termite *Termes* (*Cyclotermes*) *obesus* (Rambur) are responsible for the elaboration of cellulose destroying enzymes, cellulase and cellobiase. It is, therefore, obvious that this termite can attack sound timber.

Digestion in Various Castes

Among the various castes of the termite colony, only workers show high cellulase and cellobiase activities. The king, queen and alates (winged forms) show only slight cellobiase activities while soldiers and nymphs show neither cellulase nor cellobiase activities (Table III). This would indicate that these castes cannot digest cellulose as such, but that they derive their nutrition from one or more of three sources, *viz.*, (i) the fungus beds in the termitaria, (ii) regurgitated food of workers, and (iii) excreta of the workers. Since the

excreta will also contain the bacteria responsible for cellulase and cellobiase, it is clear that the castes other than workers which ingest excreta cannot for some morphological or physiological reasons maintain the bacteria in their digestive canal.

Extra-Cellular Nature of Enzymes

Before cellulose can be metabolised by the termite, it has to be converted first to soluble sugars like cellobiose and glucose by means of the enzymes cellulase and cellobiase respectively. Although the evidence presented by several workers is certainly suggestive of true cellulase activity, nevertheless, these workers have not demonstrated the cell-free nature of their preparations from bacteria (Greathouse, 1950). The possibility of bacterial and fungal contamination during the evaluation of enzyme activity, which often takes 24 to 72 hours cannot be entirely excluded. The results now reported in Table I show that even with the addition of bacteria isolated from the hindgut of termite and cultured in the laboratory, no increase in the activity is manifest, thereby indicating that the enzymatic activity is due to the extra-cellular enzymes derived from the bacteria harboured in the hindgut.

Action of Termite Cellulase on Cellulose Derivatives

The results obtained with the termite cellulase on cellulose derivatives show that the extent of hydrolysis is influenced by the degree of substitution of the derivatives. In so far as Cellofas TWF (a cellulose ether with degree of substitution 0·45–0·5) is concerned, the extent of hydrolysis (*vide* Table IV), when interpreted in the light of Spurlin's (1939) findings, indicates that the enzyme action has been confined to only those glucosidic linkages that lie between two unsubstituted residues. A similar finding has been reported by Holden and Tracey (1950) for the snail cellulase. In regard to the other cellulose ethers, Cellofas TAF and Cellofas TWL, the figures for the extent of hydrolysis (*vide* Table IV) do not conform to any pattern described by Spurlin (1939). This may be due to the fact that the period of enzyme action was too low for valid figures being obtained.

Inhibitors for Termite Cellulase

At 0·1 M concentration, both zinc and chromium suppress cellulase activity almost completely and cellobiase activity only partially (Table V). Further work on this aspect is likely to lead to results of practical importance.

5. Summary

(*a*) The presence of the extra-cellular enzymes cellulase and cellobiase, has been established in the gut extract of the higher termite *Termes* (*Cyclotermes*) *obesus* (Rambur).

(*b*) The activities of both these enzymes have been found mainly in the hindgut of the termite, where bacteria are mostly harboured. It appears that the enzymes are derived from these bacteria.

(*c*) Cellobiase has a sharp pH optimum at 4·0 while cellulase has an optimum pH range from 3·8 to 4·4. The optimum temperature is 30° C. and 38° C. for cellulase and cellobiase respectively. Both the enzymes are heat stable, cellobiase being more so than cellulase.

(*d*) The extent of activity of these enzymes in various castes other than workers has also been studied and a possible mechanism of digestion has been suggested.

(*e*) The action of termite cellulase on certain cellulose ethers has been studied.

(*f*) Zinc and chromium (at 0·1 M concentration) suppress cellulase activity almost completely but cellobiase activity only partially.

6. Acknowledgement

The authors wish to thank Dr. T. S. Subramanian and Mr. S. K. Ranganathan for their guidance and interest in the work. This paper is published with the permission of the Director of Technical Development and Master-General of Ordnance, Army Headquarters, New Delhi.

References

1. Beeson, C. F. C. .. *The Ecology and Control of the Forest Insects in India and Neighbouring Countries*, 1941, Forest Research Institute, Dehra Dun (India).
2. Blatt, H. A. .. *Organic Syntheses*, Collective Volume 2, 1943, John Wiley and Sons, New York.
3. Cleveland, L. R. .. *Proc. Nat. Acad. Sci.*, 1923, **9**, 424.
4. *Idem* .. *Biol. Bull.*, 1924, **48**, 289.
5. Dickman, A. .. *Ibid.*, 1931, **61**, 85.
6. Fred, E. B. and Waksman, S. A. *Laboratory Manual of Microbiology*, 1928, McGraw Hill Book Co., London and New York.
7. Greathouse, Glen, A. .. *Text. Res. J.*, 1950, **20**, 227.
8. Hagedorn, H. C. and Jensen, B. N. *Biochem. Z.*, 1923, **131**, 46.
9. Holden, M. and Tracey, M. B. *Biochem. J.*, 1950, **47**, 407.
10. Mansour, K. and Mansour-Bek, J. J. *Enzymologia*, 1937, **4**, 1.
11. Ripper, W. .. *Z. Vergl. Physiol*, 1930, **13**, 313.
12. Spurlin, H. M. .. *J. Amer. Chem., Soc.*, 1939, **61**, 2222.
13. Trager, W. .. *Biochem. J.*, 1932, **26**, 1762.

CONTRIBUTION TO OUR KNOWLEDGE OF THE PHYSIOLOGICAL ANATOMY OF SOME INDIAN HYDROPHYTES

I. The Stem of *Caesulia axillaris*, Roxb.

By M. V. Mirashi, M.Sc.

(Department of Botany, College of Science, Nagpur)

Received July 8, 1953

(Communicated by Dr. T. S. Sabnis, F.A.SC.)

Cæsulia axillaris, R., a member of the family Compositæ, is the only species of the genus *Cæsulia*, Roxb. occurring in India. It is a common marsh herb found throughout the greater part of this country in swamps, on the margins of lakes and streams and in rice fields.

The stem of this amphibious species is stout, prostrate or suberect and succulent. The surface is glabrous and striate.

Anatomical investigation of the stem reveals the following features:

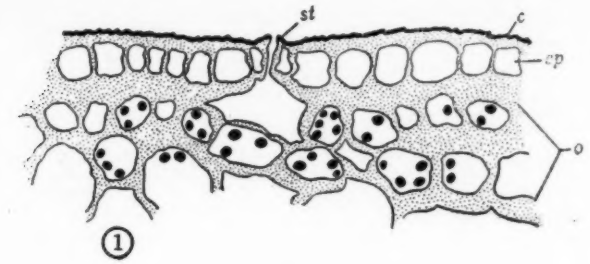
1. *Epidermis*.—A single layer of small thick-walled cells. Cuticle thin and finely papillose. Stomata in level with the ordinary epidermal cells, or slightly raised (Fig. 1).

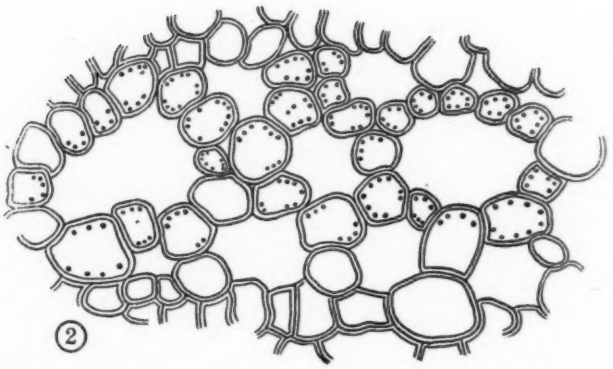
Another feature of the epidermis is the presence of anthocyanin. The pigment is generally restricted to a few epidermal cells here and there, appearing as red streaks on the external surface of the stem. Occasionally, however, the entire epidermis may come to possess anthocyanin so that externally the stem appears red.

2. *Cortex*.—This shows three concentric zones. The outer cortex consists of two to three layers of collenchyma. In the middle cortex there are well-defined schizogenous lacunæ bounded by thickwalled parenchymatous cells. The cells of the outer and middle cortex contain chloroplasts (Figs. 1 & 2).

The inner cortex, which is thinwalled and parenchymatous, shows the presence of resin-ducts placed at regular intervals. The endodermis is wavy and contains small starch-grains (Fig. 3).

Some of the cortical cells show the presence of anthocyanin which may extend even to the inner cortex.





TEXT-FIGS. 1–2. *Cæsulia axillaris*, R. Fig. 1. Part of the T.S. of the stem showing the thin papillose, cuticle (*c*), stoma (*st*), thickwalled epidermis (*ep*), and collenchymatous outer cortex (*o*), ×225. Fig. 2. Part of the T.S. of the stem showing the chlorenchymatous lacunar middle cortex, ×100.

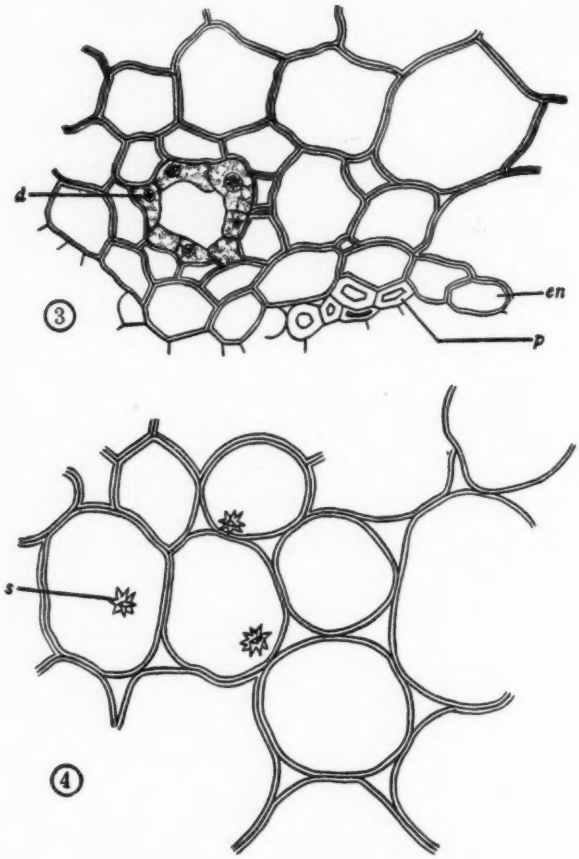
3. *Pericycle.*—Heterogeneous; radially alternating zones of thin-walled parenchyma and sclerenchymatous fibres, the latter forming crescent-shaped stereid bundles on the outer periphery of the phlœm groups of the vascular bundles.

4. *Vascular system.*—This is of the *Helianthus annuus* type. A single ring of vascular bundles separated by medullary rays. Secondary growth conforms to the sunflower type.

5. *Pith.*—Extensive; thinwalled parenchymatous cells containing druses of calcium salts (Fig. 4).

DISCUSSION

1. The stem of *Cæsulia axillaris*, R. is constructed on a fundamental plan typical for the herbaceous species of the Compositæ. It shows, at the same time, marked adaptation to the marshy habitat.



TEXT-FIGS. 3–4. *Cæsulia axillaris*, R. Fig. 3. Part of the T.S. of the stem showing the inner cortex. *d*, resin duct; *en*, endodermis; *p*, schlerenchymatous fibres of the pericycle, ×225. Fig. 4. Part of the T.S. of the stem showing pith cells containing druses of calcium salts (*s*), ×225.

2. The cortex is the most characteristic region of the stem. The outer cortex forms a firm peripheral shell of collenchyma. The lacunar middle cortex, so typical of the helophytes, functions as the aerating tissue.

3. The typical combination of semi-xerophytic or mesophytic characters with the hydrophytic ones found in the anatomy of marsh plants is evinced here by the presence of the thickwalled epidermis and cuticle, the development of an assimilatory tissue in the form of the chlorenchymatous

outer and middle cortex and the general thickness of the walls of the cortical cells. This thickness is found to increase with the decrease of moisture in the soil.

4. The presence of anthocyanin in the epidermis and cortical cells is open to various interpretations. At the present stage of investigation, the author is inclined to believe that the pigment offers protection to the plant against too strong light and high temperature. It may be mentioned here that during his investigation of the hydrophytes of Nagpur, the author has noticed several hydrophytes which develop anthocyanin on the onset of drier and warmer conditions of the summer season.

5. The foregoing account of the anatomy of the stem shows that it has efficient mechanical and conducting systems.

6. As far as the author is aware, this is the first account of the physiological anatomy of a marsh plant belonging to the Compositæ from India. Arber (1920) has drawn attention to the fact that the existence of hydrophytic members in the Compositæ is itself significant, in view of the history of Angiosperms. How far these hydrophytes have adapted themselves to their comparatively recent watery habitat is worth investigation. The present contribution is intended to be a step in that direction.

ACKNOWLEDGEMENT

The author is deeply indebted to Dr. T. S. Sabnis, D.Sc., F.A.Sc., I.A.S., for his kind interest in the work, valuable suggestions and helpful criticism.

LITERATURE CITED

Arber, A. .. *Water Plants*, Cambridge, 1920.

A NEW MEMBER OF THE EUGLENINEÆ, *PROTOEUGLENA NOCTILUCÆ* GEN. ET SP. NOV., OCCURRING IN *NOCTILUCA MILIARIS* SURIRAY, CAUSING GREEN DISCOLORATION OF THE SEA OFF CALICUT*

By R. Subrahmanyan

(Central Marine Fisheries Research Sub-Station, Calicut-5)

Received August 18, 1953

(Communicated by Dr. M. O. P. Iyengar, F.A.SC.)

Introduction

The presence of small green flagellates inside *Noctiluca* appears to have been first recorded by Weber and Weber-van Bosse (1890) who, however, did not observe the living material.

Delsman (1939–40) observed small green flagellates in large numbers swimming inside *Noctiluca*. He records them as a peculiarity of the tropical *Noctiluca* and that an accumulation of this *Noctiluca* at the surface imparts a green colour to the water. Further, according to him, this great quantity of *Noctiluca* is present in more or less brackish-water, quite near the coast.†

There appears to be no other record of this phenomenon nor any account dealing with these flagellates and their identity.

Occurrence

In recent months, the sea off Calicut was found coloured bright green in patches at several places. This occurred in December 1952 (10th to 13th), and again in March 1953, from the 12th onwards when it persisted for a longer period (several weeks). In December, the patches were seen extending from about three miles from the shore to about ten miles out; in March, the patches occurred nearer and extended from about a half mile to four miles from the shore. At night, it was reported, the sea glowed with a phosphorescent light in long patches extending North-South. The green discoloration of the sea water was found to be due to *Noctiluca* harbouring

* Published with the kind permission of the Chief Research Officer, Central Marine Fisheries Research Station, Mandapam Camp, S. India.

† Recently Prasad (1953) has recorded *Noctiluca* with a green flagellate inside it.

numerous green flagellates. It is well known that *Noctiluca* emits a phosphorescent light.

The patches of *Noctiluca* were also found to extend up to a depth of 36 metres; but, the individuals below the surface were noticed to be unhealthy unlike those occurring at the surface.

It may be interesting to mention here that, in the earlier years the plankton has been under study by the writer (May 1949 onwards), there was no bloom at all of *Noctiluca* (either with or without the green flagellates) during March as during this year. No *Noctiluca* was present in February and March 1950, November 1950 to April 1951 and February to April 1952. This year 1953, since the second week of March, *Noctiluca* with the green flagellates was the dominant form in the plankton eclipsing even the Diatomaceæ on several occasions, a very unusual feature. In December 1952, the bloom of '*green Noctiluca*' was poor as compared with that of March 1953.

During the occurrence of this green *Noctiluca* bloom, the salinity† of the surface water varied from 33‰ to 35·4‰. These values are more like those for normal sea-water than those for brackish-water and are comparable with the values for salinity recorded at this time of the year (which are among the highest values recorded here) during the earlier years. This is mentioned because of the fact that Delsman (1939–40) appears to emphasize the occurrence of *Noctiluca* in great quantity in more or less brackish-water near the coast.

An estimation of the quantity of phytoplankton at this time of the maximum occurrence of green *Noctiluca* by means of acetone extract (Harvey, 1934) gave a value of 5,200 to 6,800 Harvey units of pigment for approximately one metre-cube of sea-water. This value bears a close approximation to the average monthly values for phytoplankton bloom at its peak during the south-west monsoon period when the plankton consisted almost purely of Diatoms (Subrahmanyan, MSS.). It may be mentioned that the plankton now consisted almost entirely of this green *Noctiluca*.

Another interesting fact observed was that during the peak in the occurrence of this *Noctiluca*, high values were recorded for the silicates-content of the sea water, which bears a close inverse relationship to the absence of Diatoms in the catches. It has been observed (Subrahmanyan, MSS.) that a steep rise in the values for silicates, excepting the monsoon period, is more or less correlated with a fall in the quantity of the Diatom flora. For the corresponding period in the earlier years, the silicates values

† The data for salinity are from the records kept at the Sub-Station.

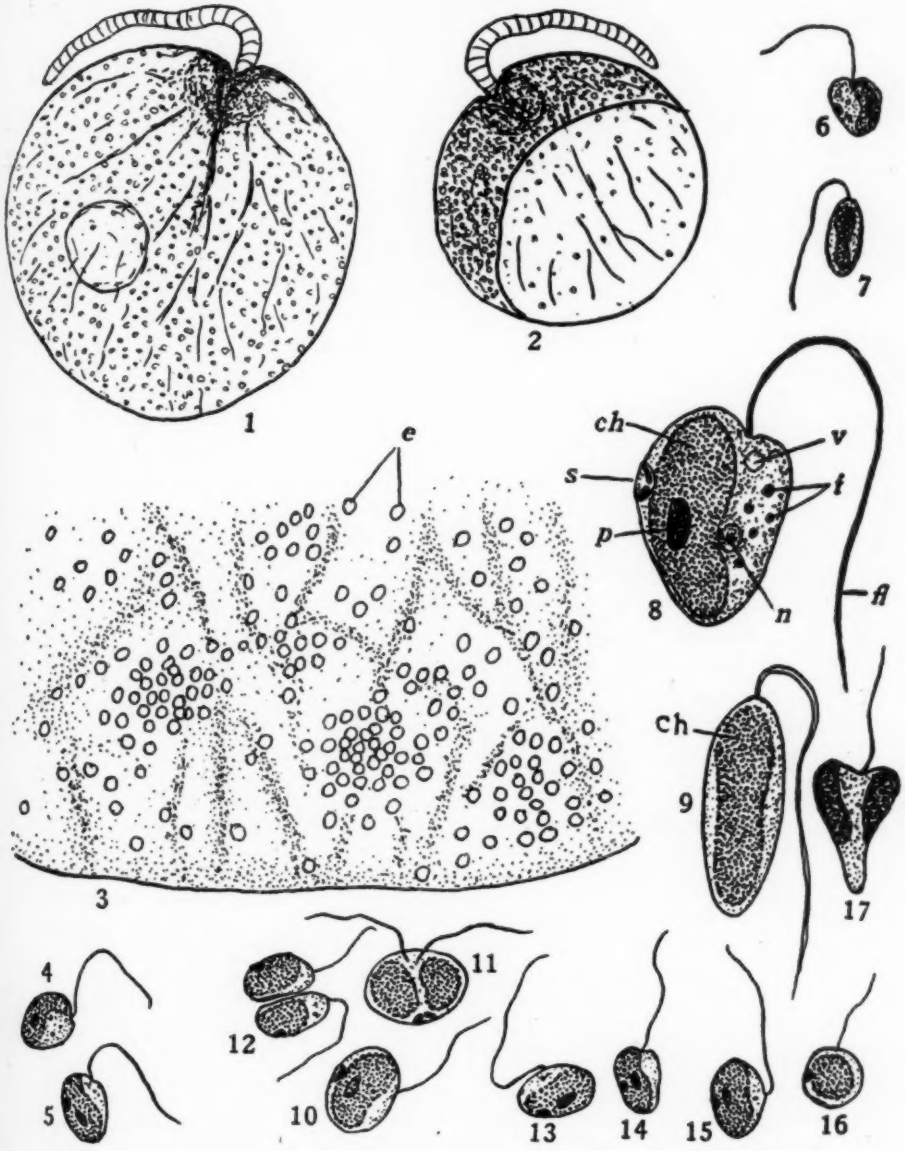
have never been so high as during this year when green *Noctiluca* occurred (recorded for the first time) dominating the plankton flora.

DESCRIPTION

The individuals of the *Noctiluca* in the plankton samples examined, showed hundreds of a tiny green flagellate swimming inside them. The flagellate was found normally uniformly distributed inside the *Noctiluca* (Figs. 1 and 3) which was alive and active as noticed by the waving of its flagellum; and, in other instances, they formed a crescent-shaped patch at the anterior end of the *Noctiluca*-cell (Fig. 2). The latter condition was more common when the plankton was crowded with *Noctiluca*, and in such instances, the *Noctiluca* also was less active or completely quiescent. The crowded samples emit an unpleasant odour when kept for some time. This leads one to infer that the conditions are not quite favourable for the flagellate. The individuals of the flagellate aggregate in large numbers near the anterior end of the *Noctiluca*-cell and then escape out and swim about in the water. Bottom samples of the plankton were invariably overcrowded and contained *Noctiluca* in the condition just described. Addition of a fixing fluid also (*e.g.*, formaldehyde) sometimes brings about this condition. Nevertheless, if a small quantity of the *Noctiluca* floating at the surface of the sample is taken and kept in a large volume of sea water, the material remains in a healthy condition for some hours. Attempts at keeping the green *Noctiluca* as such or the flagellate alone in cultures in the laboratory were not successful. At best they could be kept alive only for about six days.

When fixing agents are used, the flagellate shrinks and becomes rounded. Bouin's fluid (Gatenby and Painter, 1946), however, brings out most of its features if the fixing fluid is not allowed to act for long. The details regarding the structure of the flagellate are best studied in living individuals which have just come to rest and are quiescent.

The flagellate (Figs. 4 to 9) is pear-shaped, and is somewhat laterally compressed, the broader end being its anterior end. There is a slight invagination at the anterior end which bears a single long flagellum, which is two to two and a half times as long as the body (Fig. 8, *fl*). The narrow side-view of the flagellate is more or less elongate-elliptic (Fig. 9). The flagellate is 5 to 6 μ long, 4 to 4·5 μ broad and about 3 μ thick. It is actively motile and executes forward and generally rapid backward movements. It also often moves in a circle around a small space, rotating on its axis all the time irrespective of the nature of the movement. While moving forward, the flagellum is lashed more towards one side and while



(Figs. 1 and 2, ×80; Fig. 3, ×800; Figs. 4 to 7 and 10 to 16, ×1,700; Figs. 8 and 9, ×*c* 5,000).

moving backward, the flagellum is held more or less trailing, its distal end vibrating very actively.

The flagellate has no firm cell-wall. Its periplast is smooth. It exhibits slight metaboly while not actively swimming (Figs. 13 to 16). A bright green plate-like chloroplast is laterally disposed and, when viewed from the narrow side, it is rectangular in shape and is slightly drawn in at the middle (Figs. 8 and 9, *ch*). A conspicuous, more or less ellipsoid, body is found apposed to the chloroplast (Fig. 8, *p*). A non-contractile vacuole and a few refractile granules are seen in the hyaline portion of the flagellate (Fig. 8, *v* and *f*). A bright red eye-spot or stigma is present on one side, about the median region of the cell (Fig. 8, *s*). The stigma consists of two bright red, tiny, rod-like granules embedded as it were in a lens-shaped hyaline matrix, the granules being on the inner concave side of the lens. A tiny nucleus could be made out on careful examination (Fig. 8, *n*).

The flagellate reproduces by longitudinal division and forms two daughter cells (Figs. 10 to 12). Dividing cells are slightly larger than the active vegetative cells (Fig. 10). During division, the flagellate becomes somewhat quiescent. The chloroplast divides first and soon a second flagellum is formed close to the original flagellum (Fig. 11) and then cytokinesis is completed giving rise to two daughter-cells (Fig. 12). In the enlarged cell which is about to divide, the two granules of the stigma are seen standing out more apart than in the ordinary cells. Presumably one granule goes to each daughter-cell. Nuclear division could not be observed due to the smallness of the individual.

Microchemical tests (Johansen, 1940; Gatenby and Painter, 1946; Fritsch, 1935; Jahn, 1951) showed no presence of a cellulose wall. The conspicuous ellipsoid body lying apposed to the chloroplast (Fig. 8, *p*) showed paramylon qualities in that it did not take iodine stain, dissolved in sulphuric acid and potash and disintegrated and disappeared in formaldehyde when kept for long. The nature of the refractile globules (Fig. 8, *f*) could not be determined.

The characteristic features of the flagellate here described agree more or less closely with those of the Euglenineæ. But, the flagellate is, however, remarkably simple in its structural features as compared with the other members of the Class. The flagellate has not got a well-defined "gullet" as in the genus *Euglena*; but, the slight depression at its anterior end evidently represents the beginnings of a primitive gullet. Its chloroplast is a very simple plate. Its periplast is smooth and is not striated. The stigma is far away from the flagellum. The flagellate is clearly a primitive member

of the group, and may be placed in a new genus by name *Protoeuglena* inside the Euglenaceæ; and the flagellate itself may be named *Protoeuglena noctilucæ* gen. et sp. nov.

DIAGNOSES

Protoeuglena gen. nov.

Pertinet ad Euglenoideas, flagellatas. Cellulæ pyriformes, lateraliter compressæ; latior anterior apex unico flagello ornatus atque invaginatione ad medium. Corpus metabolicum. Periplastum leve. Chloroplastum lucide viride, unicum, laterale. Paramylum, stigma atque unicum vacuolum non-contractile adsunt.

Protoeuglena noctilucæ sp. nov.

Pertinet ad Euglenoideas, flagellatas. Cellulæ pyriformes, corpore pardurante vita activa lateraliter compresso, 5–6 μ longitud., 4–4·5 μ latit. atque 3 μ crassit. Latior apex anterior ornatus invaginatione definita in medio, ornatus etiam unico flagello duplo vel duple et dimidio longiore quam individuum. Aspectu laterali flagellata, elongato-elliptica. Periplastum leve. Organismus tenuiter metabolicus, præsertim in statu quiescenti vel cum sub microscopio observatur per spatium longum. Chloroplastum lucide viride, unicum atque laterale; lamina oblonga tenuiter inducta ad medium complens latus aspectu laterali. Paramylum unicum, minutum atque ellipsoideum, chloroplasto appositum. Stigma lucide rubra, constans duplici virgato granulo in lenticula hyalina, granulo utroque posito in interiore latere concavo lenticulæ. Nucleus parvus. Nonnulli globuli refractiles atque vacuolum non contractile adsunt. Reproductio per divisionem longitudinalem in duas cellulas filiales.

Typus lectus in interiore parte *Noctiluca miliaris* Suriray in oris maris ad Calicut, in littore occidentali Indiæ.

Protoeuglena gen. nov.

Euglenoid flagellate. Cells pyriform, compressed laterally, broader end anterior, with a depression (invagination) in the middle and bearing a single flagellum. Body metabolic. Periplast smooth. Chloroplast bright green, single and lateral. Paramylon, stigma and a single non-contractile vacuole present.

Protoeuglena noctilucæ sp. nov.

Euglenoid flagellate. Cells pyriform, body in active life compressed laterally, measuring 5 to 6 μ long, 4 to 4·5 μ broad and 3 μ thick. Broader end anterior, with a definite depression (invagination) in the middle and

bearing a single flagellum two to two and a half times as long as the body. In side-view, flagellate elongate-elliptic. Periplast smooth. Organism slightly metabolic, particularly when stationary or when kept under observation for long under the microscope. Chloroplast bright green, single and lateral; an oblong plate slightly drawn in at the middle, filling the side when viewed laterally. Paramylon single, tiny and ellipsoid, apposed to the chloroplast. Stigma bright red, consisting of two tiny, rod-shaped granules inside a hyaline " lens " the granules being on the inner concave side of the " lens ". Nucleus small. A few refractile globules and a non-contractile vacuole present. Reproduction by longitudinal division into two daughter-cells.

Occurrence.—Recorded inside *Noctiluca miliaris* Suriray in the sea off Calicut, on the west coast of India.

DISCUSSION

It may be of interest to mention here that a few Euglenineæ have been described from the sea and they have been found to be very simple in their structure (Schiller, 1925; Fritsch, 1935). Fritsch (*op. cit.*, p. 732) states that these simple types " may well afford data as to the affinities of the whole Class ".

The present flagellate (*Protoeuglena noctilucæ*) shows a certain amount of resemblance to the marine member *Ottonia caudata* Schiller (Fig. 17) in having a slight depression (invagination) at its anterior end. It is much more primitive than *Ottonia* in having only a single lateral chloroplast whereas *Ottonia* has two lateral chloroplasts.

Pringsheim (1948, p. 52) states that " Judged by other primitive algal types, the primitive *Euglena* should have a single, more or less cup-shaped, chromatophore ". And further, he is of the view that a really primitive form has yet to be described though, *Euglena archæplastidiata* Chadefaud shows certain primitive characteristics. It would appear that the present flagellate (*Protceuglena noctilucæ*) supplies the missing link or one of the missing links. The chloroplast, in this instance, though not cup-shaped is simple enough. In fact, it may be considered more primitive to a cup-shaped one. The absence of a gullet is another feature worth noting. The small depression (invagination) at the anterior end may be considered as the beginning of a gullet. The present euglenoid appears to be the simplest member of the Euglenineæ so far known.

A few words may be added here on the relationship between the euglenoid and *Noctiluca*. It is not clear how this association is brought about.

The *Noctiluca*-cells concerned, as already mentioned, appear to be quite healthy and active. The association appears to be more of a symbiotic nature rather than of parasitism or saprophytism. The euglenoid, however, does not appear to have a free existence, for, they did not survive outside the *Noctiluca* for any length of time as became evident by keeping them in cultures. Saprophytism is ruled out; if it were so, one should expect an increase in their abundance after the " host " *Noctiluca* dies; the observations are to the contrary. How they happened to be inside *Noctiluca* could not be decided; but, they were seen to multiply by division inside them. Cases of symbiotic relation between two algæ of two different Classes have been recorded previously. For instance, the alga known as *Glaucocystis* is now considered to be a case of symbiosis between a colourless species of *Oocystis* (a green alga) and an unicellular member of the blue green algæ (*cf.* Fritsch, 1935, p. 186). The present case may, therefore, be considered a case of symbiotic relationship between a member of the Dinophyceæ and a member of the Euglenineæ.

Some experiments could have been carried out, but no *Noctiluca* free from the euglenoid was available during the corresponding period when the green *Noctiluca* occurred. Samples of plankton towards the fag-end of the green *Noctiluca* occurrence and at other times also were crowded and contained dead *Noctiluca*, and the euglenoid was found both inside the dead *Noctiluca* and also outside it in the water. But the euglenoid was pale green or yellowish in colour and presented a very unhealthy appearance, and the majority of them were non-motile and appeared to be quite dead. It was mentioned earlier that overcrowding leads to an unhealthy condition and the plankton sample emits an unpleasant odour. It would appear that some such cause or other external factors have hastened the death of the *Noctiluca* and when the *Noctiluca* becomes unhealthy and dies, the euglenoid inside it also naturally becomes unhealthy and dies. Observations are being continued for obtaining further information on these obscure factors.

Summary

A new marine member of the Euglenineæ, *Protoeuglena noctilucæ* gen. et sp. nov., is described in detail. The euglenoid occurs in very large numbers inside healthy and active *Noctiluca miliaris* Sur. individuals which consequently appear green. Such *Noctiluca* individuals occur in swarms frequently colouring the sea green in patches. The euglenoid is very simple in its structure. It is pear-shaped with a broad anterior end and a narrow posterior end and possesses a single flagellum, a single green plate-like chloroplast, a paramylon granule in close apposition to the chloroplast. And it

shows a slight depression at the middle of its anterior end. This depression (invagination) very probably represents the beginnings of a gullet. It reproduces by longitudinal division. It appears to be a very primitive member of the class. Its association with *Noctiluca* appears to be symbiotic. It is suggested that this euglenoid is a most primitive member of the Euglenineæ coming very close to the ancestral type from which the rest of the Euglenineæ have been derived.

ACKNOWLEDGMENT

I am thankful to Dr. N. K. Panikkar for his keen interest in this work, to Professor M. O. P. Iyengar for going through the typescript and suggesting improvements and to Rev. Fr. H. Santapau, S.J., for translating the diagnoses into Latin.

REFERENCES

N.B.—A list of the papers consulted in connexion with this account will be too long for inclusion here; only those directly used in the preparation of this paper are cited here.

Delsman, H. C. .. "Preliminary plankton investigations in the Java Sea," *Treubia*, 1939–40, **18**, 137–81.

Fritsch, F. E. .. *The Structure and Reproduction of the Algæ*, I. Cambridge, 1935.

Gatenby, J. B. and Painter, T. S. *The Microtomist's Vade-Mecum*, 10th Edn., London, 1946.

Harvey, H. W. .. "Measurement of phytoplankton population," *Journ. Mar. Biol. Assn., U.K.*, N.S., 1934, **19**, 761–73.

Hayes, H. L. and Austin, T. S. .. "The distribution of discoloured sea water," *Texas Journ. Sci.*, 1951. **3** (4), 530–41.

Jahn, T. L. .. *Euglenophyta* in G. M. Smith, Ed. *Manual of Phycology*, 1951, Waltham, Mass.

Johansen, D. A. .. *Plant Microtechnique*, Ist Edn., London and New York, 1940.

Lemmermann, E. .. *Euglenineæ in Süsswasser-Flora*, Heft 2, *Flagellatæ*, 1913.

Oltmanns, F. .. *Morphologie und Biologie der Algen*, I. Jena, 1922.

Pringsheim, E. G. .. "Taxonomic problems in the Euglenineæ," *Biol. Rev.*, 1948, **23**, 46–61.

Schiller, J. .. "Die planktonische Vegetationen des Adriatischen Meeres," *Arch. Protistenk.*, 1925, **53**, 59–129.

Senn, G. .. *Euglenineæ* in *Nutürl. Pflanzenfam.*, Ist Edn., 1900, I, Ia, 173–85.

Smith, G. M. .. *The Freshwater Algæ of the United States*, 2nd Edn., New York and London, 1950.

Weber, M. and Weber-van Bosse, A. "Quelques nouveaux cas de Symbiose," *Zool. Ergebn. einer Reise in Niederl. Ost. Indien*," 1890, I. (Cited from Delsman, H. C., 1939–40).

Gojdics, Mary .. *The Genus EUGLENA*, Madison, 1953 (Received too late for citation in text).

Prasad, R. R. .. "Swarming of *Noctiluca* in the Palk Bay and its effects on the 'Choodai' Fishery with a note on the possible use of *Noctiluca* as an indicator species," *Proc. Ind. Acad. Sci.*, 1953, **38**, 41–47.

Explanation of Text-Figures

Protoeuglena noctilucæ gen. sp. nov.

Text-Figs. 1–17. **Fig. 1.** *Noctiluca* with the euglenoid distributed uniformly inside; Fig. 2. *Noctiluca* with the euglenoid aggregated into a crescent-shaped band inside; Fig. 3. Portion of *Noctiluca* magnified to show the distribution of the euglenoid, *e*, inside; Figs. 4–9. The euglenoid showing structure; Figs. 8 & 9. freehand drawing to scale to show details: *v*—vacuole; *f*—refractile bodies; *ch*—chloroplast; *s*—stigma; *p*—paramylon; *n*—nucleus; *fl*—flagellum. Figs. 10–11. Division stages; in 11 the chloroplast has already divided and two flagella are also seen; cytokinesis has not taken place. Fig. 12. Daughter cells. Figs. 13–16. Euglenoid showing changes of body shape—metaboly. Fig. 17. *Ottonia caudata* Schiller (after Schiller).

A CONTRIBUTION TO THE LIFE-HISTORY OF *ARTOCARPUS LAKOOCHA* ROXB.

By I. Banerji, F.A.Sc. and A. Hakim
(*Department of Botany, Calcutta University*)

Received August 24, 1953

Artocarpus lakoocha belonging to the family Moraceæ is a tree, monœcious in habit. It is tropical in distribution and flowers towards the end of November in Calcutta. It has been reported in India from Assam, Bengal, Behar, Orissa, Madras, Uttar Pradesh and Bombay.

The earlier work on the embryology of the family has been recorded by Schnarf (1931). During recent years a few important papers have been published of which mention may be made of Subba Rao's (1940) work on *Artocarpus integrifolia* and Anantaswamy Rau's (1942) on *Strebulus asper.*

Material and Methods

The material for this investigation was obtained from a plant growing in the college compound. It was fixed in Nawaschin's and Allen's modified Bouin's fluids. Before fixation the catkins were cut into small bits and the core of the catkins were removed. The materials were dehydrated, cleared and embedded in the usual way. Sections were cut 8 to 12 microns thick and stained in Heidenhain's iron-alum hæmatoxylin.

Observations

Floral morphology.—The catkins are axillary and diœcious. The male catkins appear first. Both the catkins have a club-shaped central core around which the flowers are compactly arranged. A sterile collar is present at the base of the catkins.

The male flower is represented by a single stamen and is surrounded by the perianth which grows rapidly and arches over the stamen (Fig. 1). In the female catkin the growth of the perianth is unequal, it encloses the pistil, the style of which projects out of the flower in the mature stages (Figs. 2 and 3).

2. *The Anther and Pollen Development.*—In the initial stages of development, the anther is bilobed in appearance and consist of a mass of homogeneous cells. The archesporial cells become differentiated in the hypodermal layer and are easily made out by the rich cytoplasmic contents of the

cells (Fig. 4). They soon divide to produce a primary parietal and a sporogenous layer. The parietal layer divides to give rise to the endothecium and an inner layer, which divides by periclinal wall. The innermost layer divides again to produce the tapetal cells on the inside (Fig. 5). The two middle layers get crushed during the development of the microspores. The tapetum is of the secretory type. During the meiotic divisions of the microspore mother cells, the nuclei of the tapetal cells divide giving rise to binucleate tapetal cells. The endothecial cells show the characteristic fibrallar bands only at maturity.

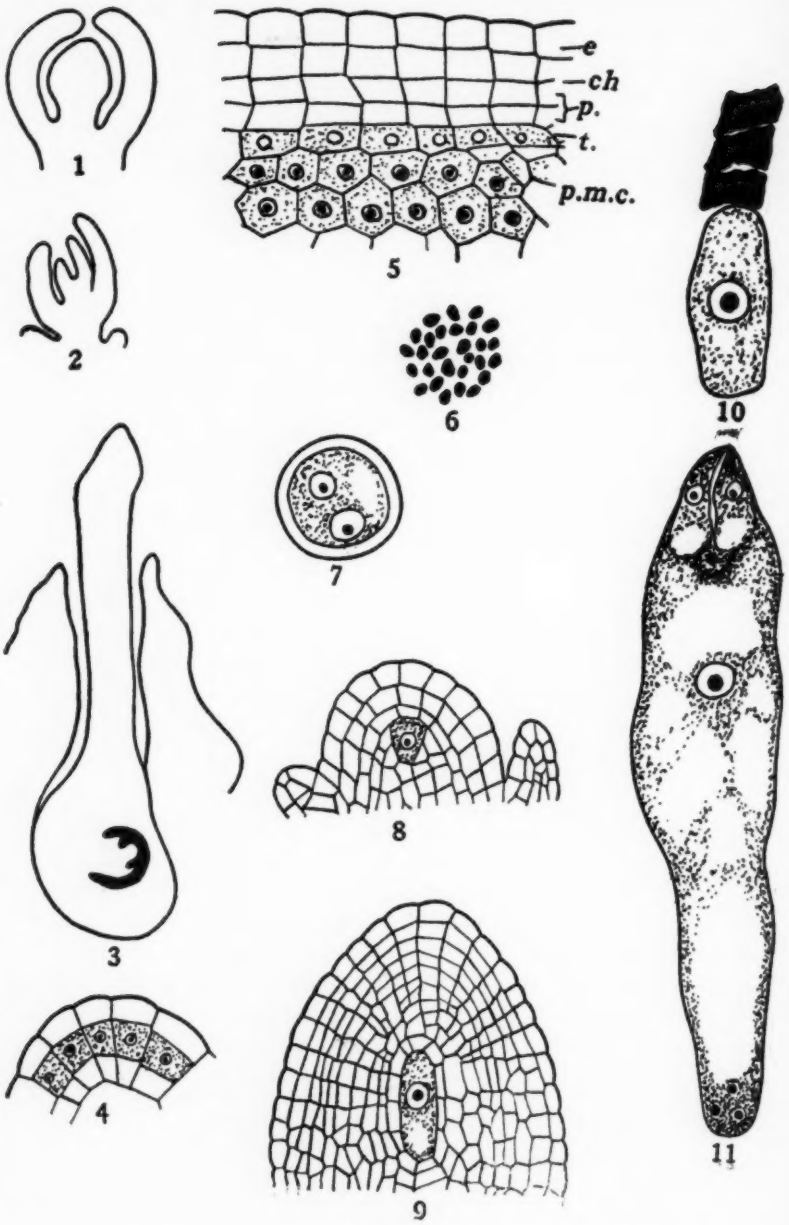
The primary sporogenous cells divide a number of times to give rise to microspore mother cells. Meiosis appears to be normal. At diakinesis the pollen mother cells round off. During metaphase of division I, 28 bivalent chromosomes are seen (Fig. 6) and the same number has been recorded during the II division. At the tetrad stage the microspores become invested by a mucilaginous substance. The microspore tetrads are mostly of the tetrahedral type and cytokinesis takes place by furrowing.

The pollen grains are spherical in outline and measure from 14·2 to 16·4 microns in diameter.

3. *Ovule and megasporogenesis.*—There is a single bitegmic crassinucellate ovule in each ovary. It arises as a lateral protuberance but soon curves and assumes an anatropous form (Fig. 3). The inner integument is the first to arise and consists of 2–3 layers of cells. The outer integument is comparatively massive and consists of 4–6 layers of cells. The inner integument gives rise to the micropyle.

The archesporial cell arises even before the origin of the outer integument in the 4th layer of the nucellus (Fig. 8). It increases in size and directly functions as the megaspore mother cell. During the reduction divisions of the megaspore mother cell, the cells of the nucellus lying above it, divide by anticlinal and periclinal walls and give rise to a nucellar cap many cells in thickness (Fig. 9). The megaspore mother cell divides resulting in two dyad cells which divide once again to produce a linear row of four megaspores. The chalazal megaspore functions while the remaining three degenerate (Fig. 10).

4. *Female Gametophyte.*—The functional megaspore increases very much in size and is seen to be capped by the remnants of the degenerating megaspores (Fig. 10). The nucleus move to the centre and divides to produce the bi-nucleate stage. At this stage the embryo-sac shows distinct polarity and there is a big central vacuole. Subsequent divisions lead to the



FIGS. 1-11

TEXT-FIGS. 1–11. *Artocarpus lakoocha.* Fig. 1. Early stage of development of male flower, ×50. Fig. 2. Early stage of development of the female flower, ×50. Fig. 3. Female flower showing the protruded stigma and the developing ovule, ×75. Fig. 4. Origin of archesporium in the anther. ×650. Fig. 5. L.S. of anther showing the tissues; *e*, epidermal layer; *en*, endothecial layer; *p*, parietal layers; *t*, tapetal layer; *p.m.c.*, pollen mother cells, ×650. Fig. 7. Pollen grain, ×1,800. Fig. 8. Differentiation of the M.M.C. at the fourth layer of the nucellus, ×650. Fig. 9. M.M.C. pushed below the nucellus due to the division of the cover cells, ×600. Fig. 10. Linear tetrad of megaspores. Upper three degenerating, lower functional, ×700. Fig. 11. Mature embryo-sac ×900.

four and eight nucleate stages. The embryo-sac increases gradually in size from the 1-nucleate stage onwards. Comparative size of the embryo-sac at different stages of growth is presented below.

TABLE I

Size of the Embryo-sac at Different Stages of Development
(size in microns)

Stage	Length	Breadth
1-Nucleate ..	48·60	12·96
2-Nucleate ..	50·76	13·42
4-Nucleate ..	65·88	22·68
8-Nucleate ..	90·00	24·68
Mature E.S. ..	113·40	28·23

It would be seen from the above table, that growth in length is far more rapid and greater than that in breadth. Besides, the maximum increase in length takes place after the 8-nucleate stage.

The synergids are pear-shaped bodies with centrally placed nuclei and basal vacuoles. The egg is situated centrally and protrudes beyond the synergids. The secondary nucleus lies in the centre of the embryo-sac, connected by cytoplasmic strands. The antipodal cells are ephemeral (Fig. 11).

CONCLUSION

Darlington and Janaki Ammal (1945) have given a comprehensive list of the chromosome numbers of plants belonging to Moraceæ. In the genus *Artocarpus*, the diploid number has been recorded as 28 for *Artocarpus cannoni* and 56 for *A. integrifolia* and *A. communis*. In the present investigation 28 haploid chromosomes have been observed and thus it clearly indicates that there is a polyploid series in the family.

In *A. integrifolia*, Subba Rao (1940) found the archesporial cell to be hypodermal in origin but the megaspore mother cell was differentiated in the 4th layer of the nucellus, while Anantaswamy Rau (1942) does not mention the origin of the archesporial cell, but states that the megaspore mother cell is noted in the 4th layer of the nucellus in *Strebulus asper*. From these observations, as well as that obtained in the course of the present study, it may be inferred that the megaspore mother cell is not hypodermal in origin, but differentiates deep inside the nucellus.

The present investigation supports the observations of previous workers and conclusively proves that the development of the embryo-sac in the family *Moraceæ* is of the *Polygonum* type. It is interesting to note, however, that in the allied family Ulmaceæ. the *Adoxa* type of embryo-sac development has been recorded in *Ulmus americana*, *U. fulva*, and in a few other species (Maheshwari, 1941).

SUMMARY

The development of the microspores is of the simultaneous type. The haploid number of chromosomes is 28.

The pollen grains are binucleate at the shedding stage.

The ovules are anatropous and bitegmic. The nucellus is massive.

The archesporial cell arises in the fourth layer of the nucellus and directly functions as the megaspore mother cell.

The development of the embryo-sac is of the *Polygonum* type. The antipodals are ephemeral.

LITERATURE CITED

1. Darlington, C. D. and Janaki Ammal, E. K., (1945) *Chromosome Atlas of Cultivated Plants*, England.
2. Maheshwari, P., (1941) ... "Recent work on the types of embryo-sacs in Angiosperms," *Jour. Ind. Bot. Soc.*, **5** and **6**, 248.
3. Rao, Subba A. M., (1940) .. "Cytology and embryology in *Artocarpus integrifolia*," *J. Mysore Univ.*, **1**, 63–73.
4. Rau, Anantaswamy, M., (1942) "Development of the embryo-sac and embryo in *Strebulus asper*," *Ibid.*, **2**, 109–14.
5. Schnarf, K., (1931) .. *Vergleichende Embryologie der Angiospermen*, Berlin.

430–54 Printed at The Bangalore Press, Bangalore City, by C. Vasudeva Rao, Superintendent and Published by The Indian Academy of Sciences, Bangalore

STUDIES ON THE BLACK ROT OF COFFEE

I. The Disease in South India and Some General Considerations*

By K. T. Mathew

(*University Botany Laboratory, Madras*)

Received May 5, 1953

(Communicated by Professor T. S. Sadasivan, M.Sc., Ph.D., F.A.Sc.)

Coffea arabica L. is cu'tivated under tropical and subtropical conditions in most of the countries of Central and South America and in many regions in Africa, Asia and Australasia; it is estimated that there are about 13·5 million acres under *Coffea*, yielding about 2·0 to 2·4 million tons of coffee seeds annually; India produces only about 16 to 22 thousand tons from about 230 thousand acres, of which about 167 thousand acres are under *C. arabica* and the rest under *C. robusta* Linden (Kuttalalingam Pillay, 1950–51).

Pellicularia koleroga Cooke (Rogers, 1943), responsible for the 'black rot' or 'koleroga' disease of coffee is also essentially an inhabitant of the tropics and subtropics; it is the only fungus affecting coffee which enjoys distribution in almost all coffee-growing countries. The fungus has been reported on coffee and other plants from Belgian Congo, Ivory Coast, Madagascar (Africa); India, Java, Malaya, New Caledonia, New Guinea, Philippines, Queensland, Straits Settlements (Asia and Australasia); Costa Rica, Cuba, Dominican Republic, Guatemala, Jamaica, Puerto Rico, Trinidad (Central America and West Indies); Mexico, U.S.A. (North America); Argentina, Brazil, British Guiana, Colombia, Peru, Surinam and Venezuela (South America) and this has been illustrated in the World Distribution Map of Plant Diseases published by the Commonwealth Mycological Institute, U.K. [Map No. 64, 1944; Pathogen : *Corticium koleroga* (Cooke) von Höhnel]; but it still remains to be settled whether all these collections apparently agreeing to the description of *Pellicularia koleroga* (*Corticium koleroga*), given by Rogers (1943), can be considered to belong to a single species. That any knowledge about this fungus and the disease it causes is of considerable interest to all coffee-growing countries is apparent. Nevertheless, it must be admitted that the fungus has never assumed the importance of *Hemileia vastatrix* B. and Br. to the old world or *Omphalia flavida* Maubl. and Rangel to many Latin American countries and does not present any serious threat

* Part of thesis approved for the Degree of Doctor of Philosophy of the University of Madras.

to wipe out coffee cultivation; it is highly destructive only under narrowly defined environmental conditions.

The author observed the disease under field conditions at the Coffee Research Station, Balehonnur, Mysore, during three seasons; he had also the benefit of answers given to a questionnaire on the disease by a number of prominent planters.

HISTORY AND NOMENCLATURE OF THE CAUSAL ORGANISM

Cooke (1876 *a*, *b*) was the first to describe the disease and the fungus which was originally collected from Mysore, under a new genus *Pellicularia*, with *Pellicularia koleroga* Cooke on coffee as the type species. In 1910, von Höhnel examined the type collection of Cooke at the Kew Herbarium; showed that Cooke's description of the fungus as a hyphomycete having sessile, globose, hyaline, echinulate spores was incorrect; described the basidia and basidiospores of the fungus; and changed its name to *Corticium koleroga* (Cooke) v. Höhnel. Coleman, Venkata Rao and Narasimhan (1923) were the first to describe the sclerotial stage of the Mysore fungus.

Several accounts of the same or similar diseases on coffee and other plants appeared from other parts of the world, but there was considerable disagreement between the different workers as to whether all the fungi were identical with the Mysore fungus (Ernst, 1880; Stevens and Hall, 1909; Kuijper, 1912; Fawcett, 1914; Burt, 1918; Butler, 1918; Wolf and Bach, 1927; Bally, 1929; Narasimhan, 1933; Briton-Jones and Baker, 1934; Matsumoto and Yamamoto, 1935; Tims and Bonner, 1942 *b*; Rogers, 1943). Burt (1918) recognised at least two distinct species among numerous collections, namely, *Corticium koleroga* (Cooke) v. Höhnel and *C. stevensii* Burt. Briton-Jones and Baker (1934), Matsumoto and Yamamoto (1935) and Tims and Bonner (1942 *b*) agreed with him. Wolf and Bach (1927) thought that there was not sufficient basis in treating these as two separate species, with which view Rogers (1943) was in agreement. Rogers who made a study of this and other closely related species felt the need for the revival of the old generic name *Pellicularia*; redescribed the genus with *Pellicularia koleroga* Cooke as the type species; grouped together many fungi previously described under the genera, *Corticium*, *Peniophora*, *Hypochnus* and *Botryobasidium* under this genus; and considered *Erysiphe scandens* Ernst, *Hypochnus ochroleucus* Noack, *Hypochnopsis ochroleuca* Noack, *Corticium ochroleucum* (Noack) Burt, *Corticium koleroga* (Cooke) v. Höhnel and *Corticium stevensii* Burt, to be synonyms of *Pellicularia koleroga* Cooke. Venkatrayan (1949) questioned the validity of the name *Pellicularia koleroga* and expressed the opinion that the coffee black rot fungus should be known as *Botryobasidium koleroga* (Cooke) Venkatrayan,

failing which its name should be preserved as *Pellicularia koleroga* Cooke emend. Rogers. Jackson (1948), however, considered the revision of *Pellicularia* suggested by Rogers as acceptable. The author has also followed the classification of Rogers.

Coffee Cultivation in South India

In South India coffee is grown under shade trees, on the slopes of hills, at altitudes varying from 1,500 to 5,500 feet above m.s.l., in Coimbatore, Coorg, Malabar, Madurai, Mysore, Nilgiris, Tirunelvely and Travancore-Cochin. *Coffea robusta* is mostly grown below 3,000 feet and *C. arabica* above 3,000 feet. The average annual rainfall in the different coffee-growing tracts ranges from about 50 to 140 inches and the temperature from 50 to 95° F. during the year. The important coffee-growing tracts of South India, showing their main features and total acreage are presented in Table I, prepared from data published by Kuttalalingam Pillay (1950–51) and Ramanathan, Bal and Thomas (1951).

Distribution of the Disease in South India

In South India black rot is severe only in those areas growing *Coffea arabica* and coming under the influence of heavy South West Monsoon rains from June to September. In Mysore the disease is found widespread in all important coffee growing tracts of Koppa, Mudigere, Bababudans, Chickmagalur, Belur, Alur and Saklaspur; it is not seen in some low rainfall areas, but is severe in certain localities. Black rot is also fairly widespread in N. Coorg and in the *C. arabica* areas in S. Coorg. The disease is found to a lesser extent in the western slopes of Gudalur-Wynaad (Nilgiris and Malabar), the Biligiris, the Anamalais (Coimbatore) and the Nelliampathies (Travancore-Cochin). The predominantly N.E.M. areas in the Pulneys (Madura), the Shevaroys (Salem), the eastern slopes of Nilgiris and Tirunelvely are practically free of the disease; so also are the *C. robusta* areas in Malabar and S. Coorg, although they are under the influence of heavy S.W.M. rains; the disease however, may be present in negligible amounts; Butler (1918) has reported its presence in the Shevaroys. The main coffee-growing districts in South India and the distribution of the disease are shown in Text-Fig. 1.

Conditions Favourable for the Disease and Its Duration

Humidity is the most important single factor which determines the occurrence of black rot in any area. The disease is prevalent only for a period of 3–4 months, during the rainy season from June to September. With the onset of the S.W.M. by about June the disease usually appears, but remains inconspicuous till it reaches the maximum during July and

TABLE I

The chief coffee-growing tracts of South India, showing some of their main features and total acreage

District or zone	Elevation in feet	Annual rainfall		Area in acres		Total area in acres
		Quantity in inches	Period	*Coffea arabica*	*Coffea robusta*	
I. Madras Presidency						
1. Coimbatore	3,000–5,500	45–70	S.W.M. and N.E.M. even	2,887	484	3,371
2. Madurai	3,000–4,000	50–70	N.E.M. heavy	25,485	625	26,110
3. Malabar	1,500–2,500	80–110	S.W.M. heavy	3,302	29,040	32,342
4. Nilgiris						
(*a*) Eastern slopes	4,000–5,700	70–80	N.E.M. heavy	21,068	1,194	22,262
(*b*) Western slopes	4,000–5,500	80–100	S.W.M. heavy			
5. Salem ..	3,800–4,600	60–80	N.E.M. heavy	10,385	nil	10,385
6. Tirunelvely ..	3,000–4,000	50–60	N.E.M. heavy	280	62	342
II. Mysore ..	2,500–5,000	55–125	S.W.M. heavy	73,216	14,059	87,275
III. Coorg						
(*a*) North Coorg	2,800–3,200	60–100	S.W.M. heavy	21,168	1,553	22,721
(*b*) South Coorg ..	2,300–3,500	70–100	S.W.M. heavy	8,144	13,543	21,687
IV. Travancore-Cochin						
(*a*) Cochin ..	3,000–3,500	100–140	S.W.M. heavy	824	915	1,739
(*b*) Travancore ..	*	*	S.W.M. heavy	*	*	*
			Total area	166,759	61,475	228 234

S.W.M.—South-West Monsoon (June to September).

N.E.M.—North-East Monsoon (October to December).

*Figures not available.

August; as the monsoon fades the disease is also reduced and fresh infections are not generally seen after September. The disease is rare or negligible in areas where the average annual rainfall does not exceed 60 inches. With increasing rains the disease is also on the increase, excessive humidity being the main requisite. The disease is, however, at its worst in areas

TEXT-FIG. 1. Shows the main coffee growing districts of South India and the distribution of 'black rot'.

where the circulation of air is very poor and where in addition to continuous rains, heavy mists are found during the monsoon. The distribution of the rain is of great importance, continuous and persistent light showers and a cloudy sky being much more favourable than heavy rains followed by intervals of bright sunshine. Under such extremely favourable conditions where rains and mist go together, black rot is equally severe under shade and in the open.

In other areas where the rain and mist are not excessive and humidity tends to be a limiting factor, the picture is slightly different. Any condition that helps to provide a cool humid atmosphere favours the disease; more disease is seen in patches under very dense and low shade; on bushes having thick foliage, which provide a suitable micro-climate within; and in low lying, water-logged, damp portions with poor drainage. In such sheltered places where these conditions are satisfied the writer has observed

stray instances of fresh infection early in May with the receipt of a few summer showers.

When the western ghats as a whole are considered, the disease is severe only on coffee grown on the western side of the ghats as observed in Mysore, Coorg and Nilgiri-Wynaad; the Pulneys and the part of the Nilgiris on the eastern side of the mountain ranges escape the disease, since the full force of the S.W.M. is not felt there. This is in evidence in the Bababudan ranges also taken as a single unit; the hills act as a barrier to the S.W.M. and the eastern slopes are comparatively free of black rot. But it is the experience of many planters situated within the western ghats that hill slopes facing east have maximum disease. This can be attributed to the greater tendency of mist to accumulate on the protected eastern side than on the wind swept western facings; the afternoon sun which is warmer also apparently checks the spread of black rot on the western aspect.

Altitude as such does not appear to be very important. The disease is present at elevations from 2,000 to 5,000 feet above m.s.l. In some places, low lying areas have more mist, while others have heavier mist at higher altitudes; the disease is always severe within the mist line.

The temperature during June to September generally varies between 60 to 75° F. in most coffee-growing areas and this is near the optimum for the rapid growth of the fungus which has been determined by Matsumoto and Yamamoto (1935) to be about 71 to 77° F.

SYMPTOMS OF THE DISEASE

The most striking feature of the disease at a glance is the presence of dark brown or black decaying leaves on the bushes, from which the name 'black rot' is derived. Closer examination reveals the presence of characteristic threads or ribbons of mycelium, running along the twigs and petioles and spreading on to the lower surface of leaves, with numerous minute clumps of hyphæ simulating sclerotia, scattered all over the dark patches. The rotted leaves although severed at the base often do not fall off and are found hanging from the branches by means of the mycelial threads (Plate IV, Fig. 2). A number of diseased leaves may also be found sticking together, where the natural disposition of the leaves help overlapping contact among them.

At the same time, on numerous healthy leaves showing no rotting, the fungus is observed to form a continuous film or pellicle showing large, dense, whitish, powdery, spore-bearing patches and when wet the whole pellicle which is entirely superficial can be easily peeled off.

Narasimhan (1933) divided the course of the disease into two distinct stages, namely the early pellicle stage and the late sclerotial stage. This conveys the impression that the fungus in the early stages is entirely superficial and only at a later stage produce sclerotia and show penetration. The writer's observation was different; both the stages were independently and simultaneously found on different leaves.

When the earliest symptoms of the disease appear at the beginning of the monsoon and also late in the season when it is fading, only the sclerotial stage is seen. The fungus on coming into contact with certain leaves form sclerotia and readily penetrate them; sclerotia are formed on leaves of all ages, berries and green shoots.

The sporogenous pellicle stage is found only at the height of the monsoon under conditions of saturated humidity. The fungus is entirely superficial during this stage and spores are not normally formed once the leaves are penetrated. On a few leaves beginning to rot the spores may be found, but, always on the green healthy portions of the leaf. Later, however, leaves showing the pellicle stage are also penetrated and begin to rot; on these the sclerotial formation is not as clear as on leaves which are directly penetrated before the formation of spores. Spores are occasionally seen on berries, but are not observed on green shoots.

Coleman *et al.* (1923) reported that in Mysore, the basidiospores are formed only for a very short duration of about 2 weeks. The author during 3 seasons (1950, 1951, 1952) at Balehonnur, observed the earliest basidiospore formation by the last week of June and on coffee and numerous other hosts the spores could be seen in abundance, though not continuously, throughout July and August and at times in September. Whenever a break in the monsoon occurred, spore formation was checked, only to appear again with a few days of continuous rains. Nonetheless, the observation of Coleman *et al.* stresses the fact that for spore formation the fungus makes rather exacting demands on the environment. Some observations on the climatic features of Balehonnur and the incidence of black rot are presented in Table II.

There are two other features which are characteristic of the disease; the definite preference the fungus has for the lower surface of the leaves, although it is capable of infecting the upper surface, as occasionally seen and its marked tendency to grow from the point of infection towards the tip of the shoots. At times when infection starts in the middle of a leaf, the fungus is seen growing down along the petiole to the shoot, but once it reaches the shoot it invariably maintains the upward course.

TABLE II

Showing the main climatic features of Balehonnur during the S.W.M. in 1950, 1951, 1952 *and the incidence of black rot*

Month	Period	Meteorological data	Year			Remarks
			1950	1951	1952	
June ..	1st to 15th	Rainfall	7·32	11·68	3·09	Light infection showing mostly sclerotial stage; sporulation infrequent, depending on intensity and distribution of rain.
		Temperature	65–83	64–83	61–83	
		Humidity	92·5	94·3	92·4	
	16th to 30th	Rainfall	4·25	6·20	10·44	do
		Temperature	64–79	65–79	64–79	
		Humidity	89·0	83·9	95·5	
July ..	1st to 15th	Rainfall	12·23	4·96	5·74	do
		Temperature	64–78	64–82	64–80	
		Humidity	93·8	93·4	96·1	
	16th to 31st	Rainfall	46·02	24·35	22·06	Severe infection and maximum sporulation
		Temperature	63–74	63–75	64–76	
		Humidity	95·6	94·1	95·0	
August ..	1st to 15th	Rainfall	14·36	14·41	11·64	do
		Temperature	64–77	60–79	63–74	
		Humidity	94·6	95·6	95·3	
	16th to 31st	Rainfall	5·75	4·99	13·44	Infection and sporulation reduced cosiderably
		Temperature	64–81	61–81	63–77	
		Humidity	94·1	85·3	90·0	
September ..	1st to 15th	Rainfall	16·36	1·54	0·90	Light infection showing sclerotial stage
		Temperature	64·76	61–81	63–81	
		Humidity	95·8	92·0	91·2	
	16th to 30th	Rainfall	4·76	8·03	2·82	do
		Temperature	63–82	65–84	64–81	The disease generally disappears by the end of the month
		Humidity	95·1	94·8	85·6	

Rainfall—Total rainfall in inches ; *Temperature*—Range of temperature in °F ; *Humidity*—average humidity in %; it may be mentioned in this connection that the meteorological data was recorded in the open; it is evident that inside the estate, under shade, the temperature should be cooler and % of humidity higher ; the data given in the table also only roughly indicate the distribution of the rain and the fluctuation in the intensity of the disease.

DESCRIPTION OF THE FUNGUS

The Mysore fungus has been described by Cooke (1876 *a*, *b*), von Höhnel (1910), Butler (1918), Coleman *et al.* (1923) and Narasimhan (1933). Cooke's description of the fungus as a hyphomycete was proved incorrect by Höhnel. The latter, describing the collection, stated that the basidia arise in clusters of 2 to 5; Miss Wakefield's drawings of the same type collection reproduced

by Burt (1918), also show a cluster of 3 basidia. But Coleman *et al.* (1923) and Narasimhan (1933) who examined fresh specimens, did not find the basidia in clusters; the author has also never observed them in clusters, although innumerable preparations were examined from a variety of host plants.

The hyphæ are hyaline when young, gradually changing to a light brown colour with age, septate, 3·2 to 8·0 μ in width and show typical rhizoctonial branching. Clamp connections and anchor cells are not seen, while hyphal fusions or anastomoses are abundant. Fructifications arise on an easily separable uniform pellicle as dense, whitish, powdery patches, often covering the entire lower surface of the leaf and showing numerous basidia and basidiospores. Basidia are simple, hyaline, oval, rounded or pyriform 5·5 to 9·7 μ in diameter, bearing four horn-shaped sterigmata of variable length, 2·5 to 11·0 μ and an equal number of hyaline, elongate spores, 5·5 to 12·4 $\times$2·8 to 4·8 μ, rounded at one end, slightly concave on one side and with a distinct apiculus at the point of attachment at the other end. Sclerotia are minute, 56 to 350 μ in diameter, pseudoparenchymatous in nature, loosely interwoven by short shells of hyphae and are found scattered all over the diseased portions of the leaves, berries and young shoots.

Others have described similar fungi from various parts of the world (Ernst, 1880; Stevens and Hall, 1909; Kuijper, 1912; Fawcett, 1914; Burt, 1918; Wolf and Bach, 1927; Rogers, 1943). Among these the descriptions of Burt (1918) and Rogers (1943) are of the entire species in general and intended to embrace the different specimens collected from widely separated regions. Table III shows the comparative measurements of different collections considered as *Pellicularia koleroga* Cooke by Rogers (1943).

Penetration of the Tissues

Some of the early workers considered the fungus as superficial and various explanations like blocking of the stomata, secretion of toxins and absorption of nutrients by osmosis were offered to account for the injury it caused (Höhnel, 1910; Butler, 1918; Coleman *et al.*, 1923). Burt (1918) for the first time reported presence of intercellular mycelium inside leaf tissues, presumably, in specimens obtained from Latin America; but failed to see evidence of penetration in the North American fungus. Wolf and Bach (1927) succeeded in showing that the North American fungus entered through the stomata, coursed between the cells and involved all tissues. Bally (1929) came to similar conclusion with the South American and Narasimhan (1933) with the Mysore fungi respectively.

TABLE III

Showing the comparative measurements of Pellicularia koleroga *Cooke made by different workers*

Name of authority	Place of collection	Name of fungus as identified by authority	Width of hyphœ in μ	Diameter of sclerotia	Diameter of basidia in μ	Length and width of spores in μ	Length of sterigmata in μ
Cooke, 1876 ..	Mysore	*Pellicularia koleroga* Cooke	*	*	*	*	*
Stevens and Hall, 1909	U.S.A.	*Hypochnus ochroleucus* Noack	5–8	*	*	7–11×4–6	*
von Höhnel, 1910	Mysore	*Corticium koleroga* (Cke.) Höhnel	6–7	*	8–11	10–12×4–4·2	2
Wakefield ..	Mysore	do	*	*	*	10–13×4–5	*
Burt, 1918 ..	Mysore, Venezuela, Puerto Rico, Colombia, Trinidad	do	4·5–6	*	10–12×7–8	10–13×3·5–5	*
	U.S.A.	*Corticium stevensii* Burt	4·5–7·5	3–4 mm.	11×7–8	8–11×3–4	*
Butler, 1918 ..	Mysore	*Corticium koleroga* (Cke.) Höhnel	5–7·5	*	11×8	10–12×4	*
Coleman *et al.*, 1923	Mysore	do	*	*	*	8–9×3·5–4·5	9–11·5
Wolf and Bach, 1927	U.S.A.	do	4·5–7	*	10–12×7–8	9–13×3·5–5	*
Narasimhan, 1933	Mysore	do	*	*	8·5–12	9·1×3·4	5–11·5
Rogers, 1943	Argentina, Venezuela, Puerto Rico, U.S.A.	*Pellicularia koleroga* Cooke	4–12	*	11·5–15(–19·5) ×8·5–10	10·5–16 ×3·5–5(–7)	6–8×2–3
Author, 1953	Mysore	do	3·2–8·0	56–350 μ	5·5–9·7	5·5–12·4 ×2·8–4·8	2·8–11·0

Microtome sections prepared during the present investigation by the writer confirmed the findings of Narasimhan (1933); sections of the pellicle stage before the rot began showed that the fungus was superficial during that period; penetration of the leaves through the stomata on the lower side and the invasion of intercellular hyphæ to the region of the palisade tissue were observed in leaves showing sclerotia and rot.

An interesting observation that has apparently escaped the notice of earlier investigators is the presence of certain crystals associated with the disease. The crystals are microscopic (often smaller than the basidiospores) and pyramidal in shape. Oxalic acid as a product of fungal metabolism has been known for a long time and De Bary (1887) reported the presence of calcium oxalate crystals in many fungi in nature; Higgins (1927) believed that oxalic acid produced by *Sclerotium rolfsii* was the principal agent of destruction. With this in mind some properties of the crystals were tested and found as follows: insoluble in water and acetic acid; soluble in HCl and HNO_3; a solution in HNO_3 in the presence of a drop of concentrated H_2SO_4, decolourises $KMnO_4$ on warming; although, some of the tests seemed to satisfy that of calcium oxalate, the identity of the crystals remains to be conclusively confirmed.

The crystals are found in abundance on the infected leaves of coffee and other plants showing the sclerotial stage and are rarely found on leaves showing the sporogenous pellicle stage; however, if immediately after the formation of the pellicle—but before the fructifications are formed—if there is a break in the monsoon for a fairly long period, numerous crystals can be found entangled in the mycelium on these leaves also; with the receipt of the next shower such leaves are generally penetrated and do not form the fruit bodies.

Spread of the Disease

The fungus mostly spreads by contact from leaf to leaf and from one bush to another, through the vegetative mycelium. The foliage of well grown coffee forms almost a continuous sheet and this is ideal for the fungus to spread by contact. Some of the infected leaves may get detached and be carried by winds to other plants at a distance, get lodged among the foliage and cause infection under suitable conditions.

Although innumerable basidiospores are formed, no large-scale infection which could be directly attributed to spread by spores was noticed at Balehonnur, during the last 3 years. The disease was generally seen in the same locality, on the same bushes year after year and new infections could almost always be traced to some original starting points, where the previous season's fungus was being harboured.

EXTENT OF DAMAGE AND ITS EFFECT ON THE HOST

The fungus is capable of infecting young shoots, leaves and berries (Plate IV, Figs. 2, 3 and 4). The nature and extent of damage caused varies considerably from place to place and year to year. In some very severe black rot areas loss of 10 to 20% of crop for the whole estate and 70 to 80% or more on individual bushes have been estimated.

While estimating the damage caused, the effect of the fungus on the vitality of the host and the resulting indirect loss also should be considered. If the leaf loss is considerable it results in severe die-back of the branches and loss of wood on which crop has to be borne next year. If by chance the bush is also susceptible to rust there is further loss of leaves and die-back of branches. The writer once observed an extreme case at Balehonnur, where a bush was attacked in July-August by the black rot fungus and in December by the ' rust ' fungus; a fair crop on the bush was allowed to ripen and this taxed the plant so much that in the absence of sufficient foliage the plant became depleted and failed to recover (Plate IV; Fig. 1).

THE LIFE-CYCLE OF THE FUNGUS

The earliest symptoms of the disease appear on those bushes that were diseased during the previous season. Under favourable conditions, infection is invariably seen to have started at places where the leaves have come in contact with branches which harbour remnants of the fungus in small cracks and crevices inside the bark. At the point of contact a fairly thick cushion of mycelium is formed, attaching the leaves to the branches and from these points the fungus spreads further. The fungus may remain active for a period of 3 to 4 months a year and during this time produces numerous basidiospores, sclerotia and vegetative mycelial threads simultaneously and spreads through their aid under suitable conditions; it has to tide over 8 to 9 months of unfavourable conditions as any one or more of these forms.

Viability of basidiospores.—Healthy leaves bearing basidiospores were collected, kept inside paper envelopes and stored at room temperature and humidity at Balehonnur. The viability of spores was determined at regular intervals. A drop of ½% agar was placed on each slide and a small portion of mycelium containing spores placed on these; the mycelium was gently tapped with a needle to dislodge the spores and then removed; and the slides were examined under a microscope to make certain that none of the spores were germinated to begin with. All slides were incubated in closed humid chambers for 18 hours. After incubation, a cover slip was placed on top of the agar on each slide and starting from one end the first 50 spores

were observed under the high power of a microscope and the number germinated determined.

On the day of collection (0-day) preparations were made from a number of leaves and from these 5 leaves giving the maximum germinated spores were selected and numbered. Material from the same leaves were used throughout and one slide was prepared from each leaf bringing the total number of spores observed each day to 250.

On the day of collection and after 1 day's storage about 90% of the spores remained viable (Table IV), but at the end of 2 days there was a steep fall in the number of germinated spores which was reduced to about 54%. Further loss of viability was gradual from the second day (54%) to the 14th day (21%). The material had become too dry by this time and the spores could not be dislodged in sufficient numbers for further observation.

TABLE IV

Showing the percentage viability of basidiospores of Pellicularia koleroga *stored at room temperature and humidity, on water agar* (July 1951)

Treatment	Replicates (leaves)					Mean
A—0 day ..	98	100	86	96	96	95·2
B—1 „ ..	96	70	96	96	96	90·8
C—2 days ..	74	34	42	68	54	54·4
D—4 „ ..	64	38	54	25	62	48·6
E—7 „ ..	46	22	48	18	78	42·4
F-14 „ ..	36	12	26	8	34	21·6

C.D. at 5% level—18; Significant by F Test $\overline{A\,B}\,\underline{C\,D\,E\,F}$

Simultaneously, the fate of the basidiospores in nature and the leaves bearing them was also observed. Fifteen apparently healthy leaves showing uniformly dense spore-bearing patches were numbered in the field and observed daily. Of these 8 leaves remained without showing rot for more than a week in spite of the fact that the lower surface was completely covered by the fungus pellicle. In the course of 10 days however, all the 15 leaves showed infection. On none of them could any trace of basidiospores be found after 10 days; the spores were shed and the basidia collapsed and the latter could not be made out from their normal shape. It appeared that the viability period of laboratory stored spores was even more than that of the spores left to nature; at any rate there is little likelihood of the disseminated basidiospores in nature surviving a long drought.

Viability of resting sclerotia.—Coleman *et al.* (1923) who first described the sclerotial stage of the Mysore fungus observed fresh infections starting from leaves showing sclerotia, attacked during the previous year and concluded that sclerotia help the fungus to tide over the dry season. However, the writer did not meet with any success in attempts to infect coffee with material collected during the previous season and containing sclerotia; a detailed account of this is given under pathogenicity studies (Table XII).

Viability of mycelial strands.—Observations in the field has convinced the author that more than the minute (50 to 350 μ), flat, loosely interwoven sclerotia and the vegetative mycelial threads found attached to dead twigs and leaves on the surface, it is the vegetative mycelium imbedded in partially dead twigs and branches (secondaries, tertiaries and occasionally primaries) that carries the fungus through the dry season and initiate fresh infections at places where the leaves come in contact with them. With the fresh infections another cycle begins.

HOSTS OTHER THAN *Coffea arabica*

The same fungus *Pellicularia koleroga* has been reported under different names on coffee and other plants, by several workers, from many parts of the world (Cooke, 1876 *a*; Stevens and Hall, 1909; Butler, 1918; Coleman *et al.*, 1923; Nowell, 1923; Weber, 1927; Marchionatta, 1927; Tucker, 1927; Wolf and Bach, 1927; Wolf, 1928; Large, 1943; Deslandes, 1944; Venkatrayan, 1947; Thomas, 1949). But some of the accounts are mere mentions of plants as collateral hosts and no information about the basis of identification and the variations to be expected in the different collections are available.

At the Coffee Research Station, Balehonnur and around its neighbourhood a number of jungle and garden plants were observed to be infected by fungi similar in appearance to that of the black rot fungus of *Coffea arabica*. These were collected during the monsoon (June to August) in 1950, 1951 and 1952. In all instances the characteristic hyphal threads were found growing along the shoots on to the leaves causing rot. Detailed microscopic examination revealed that only some of the plants were colonised by *P. koleroga*; the fungus on the others was a closely allied one and was identified as *Pellicularia filamentosa* (Pat.) Rogers.

The fungus *P. koleroga* was remarkably uniform in morphological characters on its numerous hosts and identical to that described on *C. arabica* in all respects. The very slight differences noticed in the measurements of hyphæ, sclerotia and the fruit bodies were only what could be expected in different preparations from the same host. The hosts of *P. koleroga* are listed in Table V.

TABLE V

TABLE V

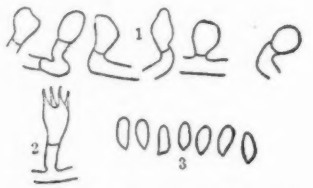
Morphological characters of Pellicularia koleroga *on various host plants found in Mysore*

No.	Name of host plant	Family	Width of hyphæ in μ		Diameter of sclerotia in μ	Diameter of basidia in μ		Length and width of spores in μ		Length of sterigmata in μ
			Mean	Range	Range	Mean	Range	Mean	Range	Range
1	*Coffea arabica* L. ..	Rubiaceæ	5·2	3·2—8·0	70—280	7·9	5·5—9·7	8·3×3·7	5·5–12·4×2;8—4·1	2·8—9·7
2	*Coffea robusta* Linden ..	..	4·9	4·0—6·4	56—224	8·4	6·9—9·7	8·7×3·8	6·9–11·0×2·8—4·1	2·8—6·9
3	*Coffea bengalensis* Roxb. ..	..	4·9	4·0—6·4	70—280	7·9	6·9—9·7	*	6·9—9·7×2·8—4·1	4·1—6·9
4	*Coffea eugenoides* ..	..	5·2	3·2—6·4	70—322	8·4	5·5—9·7	8·9×3·9	6·9–11·0×2·8—4·1	3·5—9·7
5	*Coffea abeocutæ* ..	..	5·8	4·8—6·4	56—210	*	5·5—9·7	*	6·9–11·0×2·8—4·1	2·8—5·5
6	*Coffea zanquebariensis* ..	..	5·4	3·2—7·2	70—350	8·1	5·5—9·7	9·7×4·0	6·9–12·4×3·5—4·1	3·5—6·9
7	*Psychotria thwaitesii* Hook. ..	..	5·9	4·0—6·4	*	8·6	6·9—9·7	9·0×4·0	5·5–11·0×2·8—4·1	2·8—6·9
8	*Pavetta indica* L. ..	..	5·3	4·0—6·4	70—238	8·1	6·9—9·7	8·8×3·9	5·5–11·0×2·8—4·1	2·8—5·5
9	*Pavetta breviflora* DC. ..	..	5·2	4·0—6·4	56—280	7·9	6·9—9·7	*	6·9–11·0×2·8—4·1	3·5—5·5
10	*Randia dumetorum* Lam. ..	..	4·9	3·2—6·4	70—210	*	5·5—9·7	9·0×3·8	5·5–11·0×2·8—4·1	2·8—6·9
11	*Hamelia patens* Jacq. ..	..	5·1	3·2—6·4	70—210	7·7	6·2—9·7	8·9×3·8	5·5–11·0×2·8—4·1	2·8—6·9
12	*Chasalia curviflora* Thw. ..	..	5·5	4·0—8·0	56—182	7·3	6·9—9·7	9·7×3·8	5·5–11·0×2·8—4·1	2·8—9·7
13	*Vangueria spinosa* Roxb. ..	..	5·1	4·0—6·4	56—182	8·7	6·2—9·7	8·9×3·9	6·9—9·7×3·5—4·1	3·5–11·0
14	*Mussænda* sp. ..	..	4·9	3·2—6·4	70—280	*	*	*	*	*
15	*Toddalia aculeata* Pers. ..	Rutaceæ	5·5	4·0—8·0	*	8·4	6·2—9·7	9·0×3·9	5·5–11·0×2·8—4·8	2·8—9·7
16	*Anona squamosa* L. ..	Anonaceæ	5·3	4·8—6·4	70—210	8·2	6·9—9·7	9·1×4·0	6·9–11·0×2·8—4·1	2·8—9·7
17	*Pothos scandens* L. ..	Araceæ	5·3	4·0—8·0	70—140	8·0	6·9—9·7	8·0×3·6	5·5–11·0×2·8—4·1	2·8—6·9
18	*Jasminum pubescens* Willd. ..	Oleaceæ	4·8	3·2—6·4	70—210	8·3	6·9–11·0	9·2×3·8	6·9–11·0×2·8—4·1	2·8—9·7
19	*Jasminum auriculatum* Vahl. ..	..	5·3	3·2—6·4	84—308	6·5	4·1—8·2	*	5·5–10·4×2·8—4·1	3·5—9·7
20	*Jasminum* sp. ..	..	5·3	3·2—6·4	70—308	8·1	5·5—9·7	*	8·3–11·0×3·5—4·1	3·5—9·7
21	*Dioscorea bulbifera* L. ..	Dioscoriaceæ	5·0	4·0—6·4	70—210	8·2	6·9—9·7	9·5×3·7	8·3–11·0×2·8—4·1	2·8—9·7
22	*Dioscorea appositifolia* ..	..	5·5	4·0—6·4	70—182	*	*	*	*	*
23	*Scolopia crenata* Clos. ..	Bixaceæ	5·0	4·0—6·4	70—280	7·5	6·9—9·7	8·7×3·8	6·9–10·4×2·8—4·1	3·5—9·7
24	*Croton* sp. ..	Euphorbiaceæ	5·8	4·8—8·0	*	7·9	6·2—9·7	8·9×3·9	6·9–11·0×2·8—4·1	2·8—6·9
25	*Scutia indica* Brongn. ..	Rhamnaceæ	6·4	4·8—8·0	*	*	6·2—9·7	*	6·9—9·7×3·5—4·1	2·8—6·9
26	*Clerodendron infortunatum* L.	Verbenaceæ	5·8	4·0—6·4	*	*	6·9—9·7	9·3×3·7	6·9–10·4×2·8—4·1	3·5—6·9
27	*Smilax zeylanica* L. ..	Liliaceæ	6·1	3·2—6·4	70—280	8·3	5·5—9·7	9·3×3·7	6·9–11·0×2·8—4·1	3·5–11·0
28	*Naravelia zeylanica* DC. ..	Ranunculaceæ	5·2	4·0—6·4	*	*	6·9—9·7	*	6·9–11·0×3·5—4·1	2·8—6·9
29	*Clematis gouriana* Roxb. ..	..	5·2	4·0—6·4	56—210	*	*	*	*	*
30	*Rumex nepalensis* Spr. ..	Polygonaceæ	5·7	4·0—8·0	*	8·7	5·5–10·4	*	6·2–11·0×2·8—4·1	2·8—6·9
31	*Piper* sp. ..	Piperaceæ	5·0	4·0—6·4	70—210	8·2	6·9—9·7	8·9×3·9	6·9—9·7×2·8—4·1	4·1—6·9
32	*Camellia* sp. ..	Ternstroemiaceæ	5·6	4·8—6·4	70—280	8·1	5·5—9·7	9·4×3·8	8·3–11·0×2·8—4·1	2·8—6·9
33	*Cassia fistula* L. ..	Leguminosæ	4·9	4·0—6·4	56—196	*	*	*	*	*

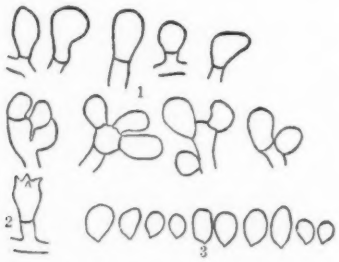
* — material not obtained; the mean values given are that of 50 readings.

In contrast, much variation was noticed among the different collections identified as *P. filamentosa*. They could be divided into two main groups, those having simple basidia and those in which basidia arose in clusters of 2 to 5; within these two groups also other variations were observed. Nevertheless, they all seemed to broadly agree with the description of *P. filamentosa* given by Rogers (1943). According to Rogers *Hypochnus filamentosus* Pat., *H. solani* Prill. and Del., *Corticium vagum* sensu Burt., *C. vagum* var. *solani* Burt., *C. vagum* subsp. *solani* (Prill. and Del.) Bourd. and Galz., *C. solani* (Prill. and Del.) Bourd. and Galz., *C. microsclerotia* (Matz) Weber, *C. areolatum* Stahel, *Oidium citri* Bondar and *Botryobasidium solani* (Prill. and Del.) Donk are all synonyms of *Pellicularia filamentosa* (Pat.) Rogers.

The collections identified by the author as *P. filamentosa* were found to differ from the coffee black rot fungus in the following respects: the hyphæ were generally thicker; no sclerotia were observed in nature; the fructifications were more coloured being buff or dirty pink; the basidia were simple as in the coffee fungus or in clusters of 3 to 5; the basidiospores were more rounded and broader and the sterigmata shorter. Details of the hosts of *P. filamentosa* are presented in Table VI. Text-Figs. 2 and 3 show camera lucida drawings of *P. koleroga* and *P. filamentosa*, respectively.



TEXT-FIG. 2. Camera lucida drawings of basidia and basidiospores of *Pellicularia koleroga*. (1) young basidia, (2) a mature basidium showing sterigmata with the basidiospores detached, (3) detached basidiospores (× 450).



TEXT-FIG. 3. Camera lucida drawings of basidia and basidiospores of *Pellicularia filamentosa*. (1) young basidia both simple and in clusters, (2) a mature basidium showing sterigmata with the basidiospores detached, (3) detached basidiospores (× 450).

TABLE VI

Morphological characters of Pellicularia filamentosa *on various host plants found in Mysore*

No.	Name of host plant	Family	Width of hyphæ in μ		Diameter of basidia in μ		Length and width of spores in μ		Length of sterigmata in μ	Remarks
			Mean	Range	Mean	Range	Mean	Range	Range	
1	*Alamanda cathartica* L.	Apocynaceæ	5·1	4·8–6·4	8·2	6·9–9·7	8·3×5·3	6·2–9·7 ×4·1–6·9	2·8–4·1	Simple basidia
2	*Telanthera ficoidia* Moq.	Amarantaceæ	6·4	4·8–9·6	8·2	6·2–10·4	9·6×5·4	6·9–12·4×4·1–6·9	2·8–3·5	do
3	*Lantana aculeata* L.	Verbenaceæ	4·9	3·2–6·4	9·6	8·3–11·0	8·8×5·5	6·9–11·0×4·1–6·9	1·4–3·5	do
4	*Erythroxylon monogynum* Roxb.	Linaceæ	4·6	3·2–6·4	8·3	6·9–9·7	*	6·9–11·0×3·5–4·1	2·1–3·5	do
5	*Dioscorea pentaphylla* L.	Dioscoriaceæ	5·1	4·0–6·4	10·0	6·9–11·7	8·6×6·0	6·9–10·4×4·1–6·9	2·1–3·5	do
6	*Dioscorea oppositifolia* L.	..	7·2	4·8–9·6	9·2	6·9–11·0	*	6·9–11·0×4·1–6·9	1·4–2·1	Basidia in clusters
7	*Dioscorea bulbifera* L.	..	7·0	4·8–9·6	8·3	5·5–11·0	9·4×6·3	6·9–12·4×4·8–8·3	2·1–2·8	do
8	*Salvia* sp.	Labiatæ	8·1	4·8–9·6	9·0	6·9–11·0	*	6·9–13·8×5·5–8·3	2·1–3·5	do
9	*Capsicum fruetescens* L.	Solanaceæ	6·8	4·8–9·6	9·1	6·9–11·0	9·0×5·7	6·9–12·4×4·1–6·9	1·4–2·8	do
10	*Bougainvillæa spectabilis* Willd.	Nyctaginaceæ	6·9	4·8–9·6	8·8	6·9–11·0	8·7×6·0	6·9–12·4×4·8–6·9	2·8–3·5	do
11	*Hibiscus rosa-sinensis* L.	Malvaceæ	7·4	4·8–11·2	9·6	6·9–12·4	*	7·6–9·7 ×4·8–6·9	2·1–2·8	do
12	*Memecylon umbellatum* Burm.	Melastomaceæ	4·7	3·2–6·4	7·7	5·5–9·7	7·0×4·8	5·5–11·0×4·1–5·5	1·4–5·5	do
13	*Bidens pilosa* L.	Compositæ	6·3	4·8–9·6	9·0	6·9–11·0	9·5×6·6	8·3–11·0×5·5–8·3	2·1–2·8	do

* The mean values given are that of 50 readings.

On some other plants, namely, *Cæsalpinia nuga* Ait. (Leguminosæ), *Alstonia scholaris* R. Br. (Apocynaceæ), *Gloriosa superba* L. (Liliaceæ), *Ceropegia elegans* Wall., *Tylophora tenuis* Bl. (Asclepiadaceæ), *Zizyphus* sp. (Rhamnaceæ), *Stephania hernandifolia* Walp. (Menispermaceæ), *Vitis discolor* Dalz. (Vitaceæ), *Clematis gouriana* Roxb. (Ranunculaceæ) and *Clerodendron infortunatum* L. (Verbenaceæ) the fructifications were not obtained but the fungus is believed to be the mycelial stage of *P. filamentosa*; fungi similar in appearance were also observed on certain undetermined hosts including some ferns.

In this connection it may be interesting to mention that one of the planters, Mr. W. Woodbridge, Bettadmane Estate, Mysore, reported the presence of fungi similar in appearance to the black rot fungus of coffee on *Erythrina lithosperma* Bl., *Ficus glomerata* Roxb. and *F. mysorensis* Heyne which are commonly grown as shade trees for coffee.

Since more than one fungus is known to attack many plants found in Mysore in a somewhat similar manner, their specific identity can be definitely established only if the perfect stage showing the basidiospores can be obtained. However, with some experience, *P. koleroga* (as it is found in Mysore) can be identified from material containing the characteristic sclerotia.

CULTURAL STUDIES

Isolation of the fungus Pellicularia koleroga

Coleman *et al.* (1923) were the first to isolate the pathogen with comparative ease, from the basidiospores. Others less fortunate, who did not obtain the fructifications in nature, have experienced considerable difficulty in isolating the fungus from diseased material showing vegetative mycelium and sclerotia (Wolf and Bach, 1927; Briton-Jones and Baker, 1934; Matsumoto and Yamamoto, 1935). Tims and Bonner (1942 *a*), however, reported considerable success in isolating the N. American fungus from young sclerotia (3 to 4 mm.), by surface sterilising them with alcoholic $HgCl_2$ (1 : 1,000 in 50% alcohol) for 2 minutes and then plating them on water agar.

Isolation by plating.—Although repeated attempts were made by the author to isolate the Mysore fungus into pure cultures by plating diseased parts of the coffee plant on agar medium, it always proved unsuccessful. Generally no growth of the pathogen was seen, while the dishes were invariably overrun by *Diplodia* spp., *Pestalotia* spp., *Fusarium* spp. and other fungi within 48 hours. Bits of leaves, twigs and berries showing the fungus

as well as separated hyphal threads and sclerotia were planted on the medium. Five bits were placed in each Petri dish.

The following general observations could be made: *P. koleroga* was not obtained from infected field material in pure cultures on any occasion. Use of either potato sucrose, carrot, onion, oat, coffee leaf extract or water agar media after surface sterilising the infected material for 15 seconds to 2 minutes with mercuric chloride 1 : 1,000, alcoholic mercuric chloride 1 : 1,000 (in 50% alcohol), 95% alcohol, silver nitrate 1 : 100 or calcium hypochlorite 1 : 14, did not help in isolating the pathogen. The berries usually yielded a higher percentage of *Fusarium* colonies; material collected in July to September showed more of *Diplodia* spp. while in the dry remnants collected from September to November *Pestalotia* spp. was the predominant form.

The methods employed by Wolf and Bach (1927) and Matsumoto and Yamamoto (1935) were also tried without success.

Isolation from basidiospores.—Small bits of leaves showing basidiospores were cut out and stuck with their lower surface downwards to the inside of lids of Petri dishes and the spores allowed to drop on agar media in the bottom dish, after Coleman *et al.* (1923). The spores generally germinated within 48 hours and these were immediately transferred to agar slants. Sometimes bacteria were also observed to grow with the spores, but no fungal contaminations were generally seen. All the isolates were further purified by cutting single hyphal tips. It often took a very long time—about 2 weeks in some cases—before any appreciable growth could be seen from the hyphal tips. But once the growth was visible, the fungus grew sufficiently rapidly to cover the slants in another week.

Isolation of Pellicularia filamentosa

These cultures were also isolated from the basidiospores and further purified by the single hyphal tip method. However, they can also be isolated by plating diseased material, with ease.

The details of all isolates used are given in Table VII.

Maintenance of stock cultures

For the first 6 months after isolation, all isolates were subcultured alternately on malt agar and potato sucrose agar. But it was observed that the sample of malt* used inhibited growth of some fungi, especially isolates of *P. koleroga*. Hence, all isolates were successively subcultured on potato

* Malt extract obtained from Government Malt Factory, Coimbatore.

sucrose agar thereafter. The medium was acidic (pH 5 to 6) and the temperature of incubation generally adopted was 21 to 23° C. After allowing 10 to 15 days growth the cultures were transferred to a cold chamber kept at 5 to 7° C. and subcultured every 2 months. Fairly large pieces of inoculum including a little agar were used in all transfers.

Considerable difficulty was experienced in maintaining some of the isolates of *P. koleroga* in a viable state. A progressive reduction in the rate and amount of growth and in the production of aerial mycelium and sclerotia was noticeable in the case of all isolates. Isolates 3, 4 and 7 have shown the least changes from their original stage. Isolates 1, 2, 6 and 9 made very

TABLE VII

Details of the different isolates used

Isolate No.	Host plant	Place of collection	Year of isolation	Specific identity
1	*Coffea arabica* ..	Balehonnur	July 1950	*Pellicularia koleroga*
2	*C. robusta* ..	do	do	do
3	*C. bengalensis* ..	do	do	do
4	*C. eugenoides* ..	do	do	do
5	*C. abeocutæ* ..	do	do	do
6	*Pavetta indica* ..	do	do	do
7	*Psychotria thwaitesii*	do	do	do
8	*Randia dumetorum* ..	do	do	do
9	*Jasminum pubescens* ..	do	do	do
10	*Scolopia crenata* ..	do	do	do
11	*Coffea arabica* ..	do	July 1952	do
14	*Lantana aculeata* ..	do	July 1950	*Pellicularia filamentosa*
15	*Bougainvillæa spectabilis* ..	do	do	do

Isolate 12 [*Corticium koleroga* (Cooke) Höhnel] and

13 (*Corticium stevensii* Burt) were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland;

16 (*Pellicularia filamentosa*) from Ramakrishnan and Ramakrishnan (1948); and

17 (*Corticium invisum* Petch) from the Tocklai Tea Experimental Station, Assam, India.

indifferent type of growth, about a year after isolation; at times they grew as rapidly and as well as the other isolates on subculturing, but on other occasions either failed to grow or after remaining without making any appreciable growth for a long time were found to become suddenly active; it appeared that these isolates were slowly synthesising some essential metabolites or enzymes necessary for their growth. Isolates 5, 8 and 10 on the other hand grew very satisfactorily for about a year and half and then abruptly

failed to grow on further subculturing. Even greater difficulty was experienced in keeping isolate 12 obtained from Baarn viable; it was obtained on two occasions, but each time failed to grow after 2 or 3 subculturings.

In a personal communication received from the Centraalbureau voor Schimmelcultures, Baarn, the following valuable information about their methods in maintaining the culture of *Corticium koleroga* was given. The fungus is grown alternately on malt agar and potato agar after neutralising the medium. The cultures are incubated at 15° C. and subcultured at 4 months intervals. By this procedure they have maintained the fungus for over 17 years without any difficulty. The chief advantage of this method seem to lie in the initial pH of the medium which is neutral and in allowing only a very slow growth of the fungus at the low temperature of 15° C.

Isolates 13, 14, 15, 16 and 17 in contrast are easily maintained in stock cultures.

Influence of temperature

Matsumoto and Yamamoto (1935) observed that the Mysore fungus (*Corticium koleroga*) grew best at 22 to 25° C., made some growth at 5 to 8° C. and did not grow above 31° C., while the N. American fungus (*C. stevensii*) grew above 31° C., but did not grow at 5 to 8° C. Wellman, Walker, Cook and Gallegly (1951), however, found that a culture of *C. koleroga* isolated at Costa Rica had an optimum temperature of 28° C. for growth; the fungus also grew well at 32° C., but growth was inhibited at 8° C. and 36° C. Many species of *Corticium* considered by Rogers (1943) to be only synonyms of *Pellicularia filamentosa* are known to grow at higher temperatures compared to the Mysore fungus (*C. koleroga*); they also generally make 3 to 5 times faster growth (Weber, 1939; Tims and Bonner, 1942 *b*).

The present investigation confirmed the above findings. Test-tubes containing 5 mm. each of potato sucrose agar were inoculated with the different isolates and incubated at 7 to 8° C., 21 to 23° C. and 31 to 32° C. All isolates (1 to 17) grew well at 21 to 23° C. Isolates 1 to 12 failed to grow at 31 to 32° C., but made some growth at 5 to 7° C., while isolates 13 to 16 grew well at the higher temperature and made no growth at the low temperature; isolate 17 grew well at 31 to 32° C. and feebly at 5 to 7° C. Rate of growth of isolates 15 to 17 at 31 to 32° C. was faster than that obtained at 21 to 23° C.

All isolates except 12 and 13 produced some kind of sclerotia in culture, especially immediately after isolation; the sclerotia in isolates 1 to 11 were minute, flattened, sub-spherical and measured 210 to 1,400 μ in diameter;

the sclerotia produced by isolates 14 to 16 were hemispherical, flattened or irregular accumulations of varying sizes, the larger sclerotia being 3 to 7 mm. in diameter, often pitted and moist; isolate 17 formed both minute and fairly large flattened irregular sclerotia. Sclerotial formation was best at 21 to 23° C. in all isolates.

Radial growth rate

All isolates were grown in 10 cm. Petri dishes containing 10 ml. each of potato sucrose agar. These were inoculated in the centre with uniform discs (3 mm.) cut with a sterile cork borer from actively growing fungal colonies and incubated at 21 to 23° C. The linear growth was measured after 48 and 72 hours incubation and the average of the maximum and minimum diameter for each colony recorded.

Isolates 1 to 13 were slow growing, isolate 12 being the slowest; none of them produced sclerotia after a week's growth. Isolates 14 to 17 made very fast growth and covered the dishes in 3 or 4 days; characteristic sclerotia were formed within a week. The results are presented in Table VIII.

TABLE VIII

Radial growth rate of isolates 1 to 17 on potato sucrose agar

Isolate No.	Growth of fungus in centimetres	
	48 hours	72 hours
1	1·0±0·1	2·0±0·1
2	0·8±0·2	1·9±0·1
3	0·6±0·1	2·0±0·1
4	0·8±0·0	1·5±0·1
5	0·8±0·1	1·6±0·3
6	0·6±0·1	1·5±0·1
7	1·0±0·2	2·3±0·2
8	0·9±0·0	1·9±0·1
9	0·8±0·1	1·9±0·1
10	1·1±0·2	2·2±0·1
11	1·2±0·1	2·5±0·2
12	0·3±0·0	0·5±0·0
13	1·5±0·0	2·3±0·1
14	4·6±0·4	Petri dish covered
15	2·3±0·6	6·3±1·2
16	2·1±0·3	8·2±0·4
17	3·7±0·5	7·8±0·8

The mean readings of 5 replicates each with their standard error are given above.

Influence of tannic acid

According to Bavendamm (1928) wood decaying fungi which are capable of decomposing both lignin and cellulose produce a dark brown zone under

and around the growing fungus, on agar media containing substances which are poly-phenolic in nature, such as tannic acid and gallic acid. Those fungi which attack only cellulose do not react in the same way. Narasimhan (1933) found that *Corticium koleroga* formed brown discolouration in advance of the growing mycelium in media containing tannic acid.

All isolates were grown in test-tubes on potato sucrose agar containing 0·1% tannic acid to see whether they all reacted similarly. The brown discolouration was observed in all isolates except isolate 13 within 24 hours.

Influence of growth substances

Pellicularia koleroga (isolate 1) does not grow on synthetic media unless minute amounts of certain growth substances are supplied (Mathew, 1952). The following experiment was designed to test whether this was true for the entire species and could be of diagnostic value in its identification.

The basal medium had the following composition: KNO_3—2 g., KH_2PO_4—1 g., $MgSO_4.7H_2O$—0·5 g., glucose—20 g. and water to make 1,000 ml.; this was solidified with 2% agar purified by treating with aqueous pyridine (Robbins, 1939). The medium was divided into two lots and to one lot malt extract was added at the rate of 200 mg. per litre. Both series were distributed into test-tubes.

Growth of isolates 1 to 12 was negligible in the controls where no malt was present, while they made good growth in the malt added series. Isolates 13 to 17 grew equally well in both the series and showed no deficiencies for growth substances.

Pathogenicity Studies

Different workers have reported varying success in pathogenicity experiments with pure cultures of *Corticium koleroga* and *Corticium stevensii* (Coleman *et al.*, 1923; Wolf and Bach, 1927; Briton-Jones and Baker, 1934; Matsumoto and Yamamoto, 1935; Tims and Bonner, 1942 *b*).

All pathogenicity experiments reported here were conducted during the heavy S.W.M. rains (July to August 1951) at the Coffee Research Station, Balehonnur. The temperature recorded at the station varied from 64 to 75° F. during these investigations and the atmospheric humidity from 93 to 97%; about 40 inches of rain were received from July 15th to August 15th. Isolates 11, 12 and 13 were not included in any of the experiments since they were obtained only at a later date.

Pathogenicity of different isolates of Pellicularia koleroga *on* Coffea arabica *and* C. robusta

The relative ability of the different isolates of *P. koleroga* to cause fresh infection was tested on *C. arabica* and *C. robusta* seedlings; the two species of coffee mentioned are economically the most important in South India. Basket plants from the nursery were used as hosts, with one seedling in each basket. The seedlings were about 6 inches tall and had developed 2 to 3 pairs of leaves. On each seedling one pair of leaves was inoculated. Uniform discs (5 mm.) cut with a cork borer from actively growing fungal colonies were used as inoculum. These were placed at the centre of the leaves on the lower side, with the mycelium touching the leaves. A small piece of moist cotton was placed on top to keep the inoculum in position and this also proved useful in retaining moisture. The controls were treated similarly with the only exception that plain agar discs were used in the place of fungal discs. The seedlings were placed under shade in propagation chambers which had glass roofing and even during heavy rains the walls and roofs of the chambers were sprayed with water 2 to 3 times daily, to maintain a high percentage of humidity.

The criterion of infection was the formation of brownish, slightly depressed, circular or irregular patches showing sclerotia on the leaves. The infection was generally observed 4 to 5 days after inoculation. One to three small patches were formed a little distance from the inoculum and they enlarged and coalesced with each other forming large patches. Characteristic sclerotia were found on the diseased areas and occasionally on the adjacent healthy portions of the leaf. Isolates 6 and 9 were less pathogenic than the rest and it is assumed to be due to the changes they have undergone while in culture for 1 year. Both *C. arabica* and *C. robusta* seedlings seemed to be equally (Table IX) susceptible, although in nature the latter is relatively resistant.

Pathogenicity of P. koleroga (*isolate* 1) *on different species and varieties of* Coffea

At the Coffee Research Station, Balehonnur, only 2 species of *Coffea* namely *C. arabica* and *C. robusta* are grown on a field scale. But a few specimen plants of various species and varieties of coffee are also grown on some parts of the estate. Among these, natural infection by *P. koleroga* was observed on *C. arabica*, *C. robusta*, *C. bengalensis*, *C. abeocutæ*, *C. zanquebariensis* and *C. eugenoides*. Other species like *C. liberica* Hiern, *C. excelsa* A. Chev., *C. stenophylla* G. Don. *C. canephora* Pierre, *C. quillou* and

TABLE IX

Pathogenicity of different isolates of Pellicularia koleroga *on* Coffea arabica *and.* C. robusta

Isolate number	Plants inoculated			
	Coffea arabica		*Coffea robusta*	
	No. of leaves inoculated	No. of leaves infected	No. of leaves inoculated	No. of leaves infected
1	6	6	6	6
2	6	6	6	6
3	6	3	6	4
4	6	4	6	4
5	6	4	6	6
6	6	1	6	0
7	6	6	6	6
8	6	4	6	6
9	6	1	6	2
10	6	6	6	4
Control ..	6	0	6	0

Period of observation—14 days.

C. uganda were found to be free from the disease. The aim of this experiment was to see whether any of these species of coffee were actually immune to the disease.

Inoculations were made on fully grown, disease-free plants in the field as seedlings of most of the species were not available for experimental work. Diseased patches generally appeared on the 4th or 5th day after incoluation on all plants. None of the species of coffee tried were found to be immune to the disease (Table X).

Cross inoculation experiments with a few isolates of P. koleroga

Isolates 1, 3, 5, 9 and 10 were used in this experiment. These were selected mainly because the hosts from which they were originally isolated, namely *Coffea arabica*, *C. bengalensis*, *C. abeocutæ*, *Jasminum pubescens* and *Scolopia crenata* could be found within the same locality; the plants were also representatives of cultivated and jungle as well as rubiaceous and non-rubiaceous plants. The inoculations were made on disease-free mature plants in the field, during heavy rains.

Infection was generally observed from the 5th day after inoculation on all plants. All the isolates tested infected their respective hosts and also

TABLE X

Pathogenicity of P. koleroga *(isolate* 1) *on different species and varieties of Coffea*

Plants inoculated	Number of leaves inoculated	Number of leaves infected	Number of controls	Number of controls infected
Coffea arabica var. *kent* ..	4	4	4	0
C. arabica var. *coorg* ..	4	3	4	0
C. arabica var. *maragogype* ..	4	4	4	0
C. arabica var. *baramasi* ..	4	3	4	0
C. robusta ..	4	4	4	0
C. eugenoides ..	4	4	4	0
C. abeocutæ ..	4	4	4	0
C. bengalensis ..	4	4	4	0
C. zanquebariensis .	4	4	4	0
C. stenophylla ..	4	4	4	0
C. canephora ..	4	4	4	0
C. excelsa ..	4	4	4	0
C. liberica ..	4	3	4	0
C. uganda ..	4	3	4	0
C. quillou ..	4	4	4	0

all the other plants. Isolate 9 showed less infection on the different species of *Coffea*, when compared to the other isolates (Table XI).

TABLE XI

Cross-inoculation experiments with a few isolates of P. koleroga

Plants inoculated	Number of leaves inoculated					Number of leaves infected					Number of controls	Number of controls infected
	Isolate number					Isolate number						
	1	3	5	9	10	1	3	5	9	10		
Coffea arabica ..	4	4	4	4	4	4	4	4	2	4	4	0
C. bengalensis ..	4	4	4	4	4	4	4	4	1	2	4	0
C. abeocutæ ..	4	4	4	4	4	3	4	4	1	4	4	0
Jasminum pubescens ..	4	4	4	4	4	4	4	4	4	4	4	0
Scolopia crenata ..	4	4	4	4	4	4	4	4	4	3	4	0

On *Jasminum pubescens* and *Scolopia crenata* in addition to the infected patches showing sclerotia, the fungus formed the characteristic hyphal threads.

But in none of the pathogenicity experiments the fructifications were formed and hence the fungus could not be got back into pure culture.

Pathogenicity trials with infected field material of Pellicularia koleroga *on* Coffea arabica

The purpose of this experiment was to understand the nature of spread of the disease in the field and the form of survival of the fungus during the unfavourable conditions. Both fresh material from the field and 1-year old material collected during the previous season and stored in paper envelopes were used as inoculum; in addition to the controls (no inoculum) a pure culture of the fungus was also used as a check. The experiment was conducted simultaneously in the field on a mature *Coffea arabica* bush and on excised healthy leaves from the same plant kept in humid chambers. The details of the various treatments tried and the results are given in Table XII.

TABLE XII

Showing the relative ability of fresh material and material collected during the previous season to cause new infection

Inoculum			No. of leaves inoculated	No. of leaves infected
Description	Nature	Surface		
I. FIELD EXPERIMENT—				
(a) Leaves with basidiospores ..	Fresh	About 4″×1·5″	8	8
do	1-year old	do	8	0
(b) Leaves with sclerotia ..	Fresh	do	8	8
do	1-year old	do	8	0
(c) Twigs with hyphal threads ..	Fresh	About 1·5″×0·1″	8	2
do	1-year old	do	8	0
(d) Separated hyphal threads ..	Fresh	do	8	0
do	1-year old	do	8	0
(e) Pure culture of isolate 1 ..		5 mm. disc.	8	8
(f) Control (No inoculum) ..			8	0
II. EXPERIMENT WITH EXCISED LEAVES				
(a) Infection drop of spores ..	Fresh	Thickly sown	4	0
do	do	Thinly sown	4	0
(b) Infection drop of sclerotia ..	Fresh	Thickly sown	4	0
do	do	Thinly sown	4	0
do	1-year old	Thickly sown	4	0
do	do	Thinly sown	4	0
(c) Separated hyphal threads ..	Fresh	About 1·5″×0·1″	4	0
do	1-year old	do	4	0
(d) Pure culture of isolate 1 ..		5 mm. disc.	4	4
(e) Control (no inoculum) ..			4	0

When fresh material was used successful infection was obtained when a large surface of the fungus was in contact with the leaf, while 1-year old material in no form was able to initiate fresh infections; and curiously enough

maximum success was obtained with the pure culture, although the size of the inoculum was comparatively small.

Cross-inoculation with some isolates of Pellicularia koleroga, P. filamentosa *and* Corticium invisum

P. koleroga and *P. filamentosa* are both unspecialised parasites and are known to infect a large number of plants inoculated (Wolf and Bach, 1927; Matsumoto and Yamamoto, 1935; Weber, 1939; Tims and Bonner, 1942 *b*; Ramakrishnan and Ramakrishnan, 1948); *C. invisum* has also a wide host range (Tunstall and Sarmah, 1940).

The experiment was conducted inside propagation chambers described earlier, on young plants raised in baskets or pots. One pair of leaves on each plant was inoculated.

As far as possible the pathogenicity of each of these isolates on the respective hosts from which they were isolated as well as the possibility of their infecting the hosts of the other closely allied fungi, was investigated in this experiment. Isolates 1, 2, 14 and 17 have been isolated from *Coffea arabica*, *C. robusta*, *Lantana aculeata* and *Camellia theæ* respectively and all the 4 hosts were used. Instead of *Maranta arundinacea*, the original host of isolate 16, *Canna indica* L. which was shown to be very susceptible to the fungus (Ramakrishnan and Ramakrishnan, 1948) was used. Isolate 15 was isolated from *Bougainvillæa spectabilis*, but this host was omitted since young plants were not available. The results are presented in Table XIII.

Isolates 14, 15 and 16 (*P. filamentosa*) caused infection in 2 to 3 days on all hosts and formed large irregular brown patches. They proved to be the most destructive and in some cases formed the characteristic threads of hyphæ from which the detached leaves were found hanging just like in nature. Isolates 1 and 2 (*P. koleroga*) were also pathogenic on all hosts tried, but it took 6 to 8 days for the patches to appear; numerous minute sclerotia were formed on and around the infected areas. Isolate 17 (*C. invisum*) was highly destructive on *Camellia theæ* and *Canna indica*; it was less pathogenic on the two species of *Coffea* and did not infect *Lantana aculeata*. The infection spots arose as numerous, minute, irregular, brownish dots or patches all over the surface in about 5 to 7 days and later coalesced to cover larger areas.

Control of the disease

The spraying of coffee in South India against fungus diseases have been reviewed by Mayne, Narasimhan and Sreenivasan (1933) and Ramanathan,

TABLE XIII

Cross-inoculation with some isolates of Pellicularia koleroga, P. filamentosa *and* C. invisum

Isolate number	Plants inoculated									
	Coffea arabica		*Coffea robusta*		*Lantana aculeata*		*Canna indica*		*Camellia thea*	
	No. of leaves inoculated	No. of leaves infected	No. of leaves inoculated	No. of leaves infected	No. of leaves inoculated	No. of leaves infected	No. of leaves inoculated	No. of leaves infected	No. of leaves inoculated	No. of leaves infected
1	6	6	6	6	6	5	6	2	6	6
2	6	6	6	6	6	6	6	4	6	6
14	6	6	6	6	6	6	6	6	6	6
15	6	6	6	6	6	6	6	6	6	6
16	6	6	6	6	6	6	6	6	6	6
17	6	2	6	1	6	0	6	6	6	6
Control	6	0	6	0	6	0	6	0	6	0

Bal and Thomas (1951). By combining cultural methods which include proper sanitation, good drainage, free circulation of air and light, regulation of shade, removal of diseased portions by pruning and handling and centering of the bushes with one or two pre-monsoon sprayings of Bordeaux mixture (2–2–40) according to severity, the disease is successfully controlled.

DISCUSSION

The fungus *Pellicularia koleroga* is widely distributed throughout the tropical countries of the world. A warm, humid atmosphere is essential for the growth of the fungus and in all countries its activity is restricted to the rainy seasons. In South India the disease is found generally in those areas where heavy S.W.M. rains occur from June to September and is most conspicuous at the height of the monsoon in July and August (Tables I, II).

A striking feature about the fungus is that, provided the environmental conditions are suitable, it is able to infect a wide variety of unrelated plants belonging to several families. This unspecialised, multivorous nature is characteristic of *P. koleroga* and the other closely related species *P. filamentosa* (Tables V, VI). However, in Mysore, a partiality for rubiaceous plants was noticed in the case of *P. koleroga* and out of a total of 33 plants recorded

by the writer as collateral hosts of the fungus, 14 belong to the Rubiaceæ family; Coleman *et al.* (1923) recorded 8 hosts of the fungus including coffee from Mysore, of which 6 were rubiaceous plants.

The fungus is also unspecialised in the matter of selection of the host organs it attacks and is found to infect all green parts including young shoots, leaves and berries.

The fungus establishes on its hosts in two distinct forms. One is the sclerolial form in which the fungus penetrates the leaves, berries and shoots and numerous minute clumps of hyphæ in the nature of sclerotia are found scattered all over the diseased patches. The other known as the pellicle stage occurs only under highly conducive environmental conditions and the formation of basidia and basidiospores which takes place during this stage is generally restricted to the leaves; during this stage, the fungus exhibits a remarkable degree of s pecalisation and there is a delicate balance between the host and pathogen. Unlike the sclerotial stage in which the fungus is actually saprophytic on the tissues it penetrates and kills, in the pellicle stage the fungus remains superficial and apparently derives certain nutrients essential for spore formation from the living hosts. According to Arens (1929, 1934) carbonates of potassium, calcium and other salts diffuse out of the plant body into the traspiration stream and a portion of these salts are gradually dissolved in the drops of water remaining on the leaves for long period, while phosphatides, growth substances, etc., diffuse in infinitesimal amounts directly from the leaf surface. Recent work by Brian, Wright, Stubbs and Way (1951) has shown that antibiotics like griseofulvin added in minute amounts can be taken into plants through the root system and can then be translocated into the leaves and detected in the guttation fluid. It is possible that certain growth substances which are extremely active, easily volatile and essential for spore formation are derived by the fungus from the living host and that this is made unavailable once the balance between the host and pathogen is upset by the penetration and destruction of the tissues.

But what is difficult to understand is as to what makes the fungus—under the same environmental conditions—readily penetrate certain tissues while it prefers to remain superficial on others for long periods. During the pellicle stage although the fungus is found covering the entire leaf surface and is in intimate contact with it, does not penetrate the leaves for a long time; similarly, the mycelial threads that run along the green shoots are often found to cover long distances without causing any injury to the host; but at the same time, other leaves, shoots and berries are readily infected

even though only a small portion of the host organ comes in contact with the fungus.

The presence of certain unidentified crystals associated with the rotting tissues suggests that the fungus produces certain secretions in excess (the excess being converted into insoluble crystals) which either through their toxic action or by merely providing a pH suitable for the increased infective ability of the parasite, aid in the penetration of the tissues. The blackening of the tissues occurs only after they are penetrated and Narasimhan (1933) suggested that it is the secretion of oxidase by the fungus that penetrates the tissues that is responsible for the action. The nature and mode of action of the secretion or secretions of the fungus remains to be determined, but their effect is readily observed. They are very rapidly destructive and the author has observed leaves remaining apparently healthy in the evening turned completely black by the next morning.

The fungus spreads mostly through contact from one plant to another by means of the vegetative mycelium. Under normal conditions, spread of the disease by basidiospores is not as severe as one would expect. Gäumann (1950) while discussing the reasons why *Polyporus formentaris* does not cause any serious disease in Central Europe gave the following explanation. " In the tinder fungus (*Polyporus formentaris*) its one fructification produces in one vegetative season approximately 9 to 18 billion basidiospores and this may be repeated annually for 50 or 100 years. Still it is a rare disease in Central Europe. It would seem that its spores besides quickly losing their power of germination must make very special and narrowly defined demands on the environment for their development. They appear to have a high numerical threshold for infection and their requirements for successful attack are so infrequently realised." Hawker (1950) has quoted Buller's calculation that 1,000,000,000,000 spores of *Polyporus squamosus* are wasted for every one successfully established on a suitable substrate. A somewhat similar explanation seems to be acceptable for the limited spread by basidiospores observed in *P. koleroga* at Balehonnur, during the last 3 years.

Admittedly, spread of the disease by basidiospores does take place on some of the highly susceptible hosts under narrowly defined environmental conditions; otherwise it will be difficult to explain its presence in disconnected patches in the same estate and the wide distribution it enjoys on cultivated and jungle plants in many of the discontinuously distributed coffee growing tracts of South India. This opinion is further supported by field observations and results of some pathogenicity experiments. A closely allied fungus *P. filamentosa* which infects a number of plants round about the coffee

plantations at Balehonnur, has been observed only a few feet away from the coffee bushes and once on *Bidens pilosa* right under a coffee plant. But the writer has not observed any infection caused by this fungus in nature on coffee* although inoculation experiments with pure cultures (Table XIII) showed that this fungus was more destructive on coffee than even *P. koleroga*. *P. filamentosa* caused severe infection on both *Coffea arabica* and *C. robusta* in 2 to 3 days while it took 5 to 7 days for *P. koleroga* to cause infection under identical conditions. Similarly, certain species of *Coffea* found to be generally free of black rot in nature, could be readily infected with pure cultures of the fungus in inoculation experiments (Table X). The failure of *P. filamentosa* to parasitise *Coffea* spp. in general and the failure of *P. koleroga* to infect some species of *Coffea* in nature is probably due to the density of spore inoculum to cause infection in those cases being insufficient. According to Gäumann (1950) the minimum density of spore inoculum necessary for successful infection is known as the numerical threshold of infection and this value depends on the infective ability of the parasite and the resistance of the hosts; in susceptible hosts this value is low; by increasing the quantity of inoculum there is a mass action due to the localised effect of the enzymes secreted from numerous hyphæ and the additive effect of their toxic secretions induce a local breakdown of host resistance. This may explain why the vegetative mycelium of *P. filamentosa* and *P. koleroga* readily infect many plants which their spores do not seem to be able to.

Based on these assumptions it is possible to recognise the following types of susceptibility in plants to *P. koleroga*, under highly suitable environmental conditions.

Highly susceptible—the most common hosts in nature; *e.g.*, *Coffea arabica*, *C. eugenoides*, *Pavetta indica*, *Randia dumetorum*, *Scolopia crenata*, *Jasminum* spp.; infection through vegetative mycelium very easily and through basidiospores under favourable conditions.

Moderately susceptible—the less common hosts in nature; *e.g.*, *Coffea robusta*, *C. abeocutæ*, *Clerodendron infortunatum*; infection through basidiospores less frequent.

---

* On one occasion *P. filamentosa* was observed to cause severe infection on some *C. arabica* seedlings in the nursery; but the infection was readily traced to some diseased weeds that had got mixed up with the compost from which the fungus was found to grow along the stem on to the leaves. This was further proof that *P. filamentosa* can infect coffee provided the vegetative mycelium came in contact with it. A pure culture of the fungus was isolated and in pathogenicity trials both coffee seedlings and mature coffee bushes in the field were shown to be equallly susceptible.

Apparently resistant—infection rare or absent in nature but possible through the vegetative mycelium; *e.g.*, *Coffea liberica*, *C. canephora*, *Camellia theæ*, *Lantana aculeata*.

Highly resistant or immune—those plants which the fungus is not able to infect under any circumstances (No effort was made to determine such immune hosts).

Observations in the field have shown that the fungus tides over the unfavourable season (8 to 9 months) as vegetative mycelium in localised infection foci on branches and " from the epidemiological standpoint, vegetative mycelium overwintering inside the host is by far the most efficient way " (Gäumann, 1950).

There is still disagreement about the classification and nomenclature of the ' koleroga ' fungus. Burt (1918), Briton-Jones and Baker (1934), Matsumoto and Yamamoto (1935) and Tims and Bonner (1942 *a*) consider *Corticium koleroga* and *C. stevensii* as two distinct species and the presence of fairly large sclerotia both in nature and cultures of *C. stevensii* to be of diagnostic value. Wolf and Bach (1927) who first suggested that there was not sufficient basis for considering them as two separate species, however, did not find sclerotia to be either a constant or dependable character in cultures of *C. stevensii*. A pure culture of the fungus obtained from Baarn also did not produce sclerotia in cultures. Rogers (1943) agreed with Wolf and Bach, but also accepted the original name *Pellicularia koleroga* proposed by Cooke (1876 *a*) and reduced to synonymy *C. koleroga* and *C. stevensii*. Rogers has rightly pointed out that the presence or absence of sclerotia must be regarded as untrustworthy, at least until more comparative studies are carried out.

Admittedly a few of the comparative experiments with pure cultures reported in this paper have shown that *C. koleroga* and *C. stevensii* are physiologically very much different; they have different cardinal temperatures for growth; different growth substance requirements; and react differently to presence of tannic acid in the medium (Pages 22 to 25). But even a fungus isolated at Costa Rica and identified as *C. koleroga*, has very different cardinal temperatures for growth compared to the Mysore fungus (Wellman *et al.*, 1951). It is very likely that cultures of fungi identified as *C. koleroga* from other parts of the world would also show many differences; only a comparative study of as many pure cultures of these apparently identical fungi as possible, from different parts of the world can settle whether physiological characters in pure cultures can be considered dependable in the identification

of the species; such an investigation would be very desirable. Till such information is available it is best to retain the classification of Rogers (1943) for the genus *Pellicularia*, which is based on morphological characters found in nature, since the creation of more and more species based on insufficient data can only add to the confusion already present. The author is in complete agreement with Rogers and Jackson (1943) who dealing with the synonyms of American Thelephoraceæ and other resupinates have stated " Further study of some of the species included may reveal that we have erred on the side of ultra-conservatism and it may later be found desirable, when more abundant material is available for study to restore to specific rank certain species which are treated as synonyms."

SUMMARY

The occurrence of *Pellicularia koleroga* Cooke which causes the black rot of coffee has been reported from almost all the tropical countries of the world. In South India the disease is severe only in those areas coming under the influence of heavy S.W.M. rains from June to September. Humidity is the most important single factor which determines the presence of the fungus in any area.

The fungus establishes on its hosts in two distinct forms, *viz.*, the sclerotial form which shows penetration and rotting of the tissues and the pellicle stage which bears the basidiospores and where the growth of the fungus is entirely superficial. The pellicle stage is formed only on healthy tissues under conditions of relatively high humidity.

Young shoots, leaves and berries are infected by the fungus.

The fungus enters the leaves through the stomata, is intercellular and involves all tissues. Certain unidentified crystals formed through fungal metabolism are found associated with the rotting leaves and it is believed that the secretion or secretions help the fungus in the penetration of the tissues.

The disease spreads mostly through contact from plant to plant by means of the vegetative mycelium. Under narrowly defined environmental conditions spread of the disease by means of basidiospores takes place.

The spores remain viable for only a short period. The fungus tides over the unfavourable season by means of vegetative mycelium in localised infection foci on branches.

P. koleroga is unspecialised and plurivorous in nature and under favourable environmental conditions, is capable of infecting a large number of

plants belonging to different species, genera and families, although a preference for rubiaceous plants is noticed in Mysore.

Pure cultures of the fungus were isolated from leaves of a few plants showing basidiospores. All isolates were found to be physiologically and morphologically alike. They were all slow growing, had more or less the same rate of growth and produced a brown discolouration on media containing tannic acid. None of the isolates grew on synthetic media unless certain growth substances were supplied and all of them had the same cardinal temperatures for growth.

All the isolates were found to be pathogenic on species of *Coffea* and other plants inoculated.

A closely allied fungus *P. filamentosa* was even more unspecialised in the matter of host selection and was observed on a number of plants. In culture the fungus made 3 to 5 times faster growth than *P. koleroga*, grew well at comparatively high temperatures and exhibited no growth substance deficiencies. The fungus readily and rapidly infected many plants inoculated, including *Coffea* spp. However, the fungus is not generally observed causing disease on coffee in nature.

The classification of the genus *Pellicularia* proposed by Rogers (1943) is adopted.

Acknowledgements

I am very grateful to Professor T. S. Sadasivan, Director, University Botanical Laboratories, Madras, and to Dr. C. V. Subramanian, Senior Lecturer, for their guidance, help and criticism during the course of the investigations and in the preparation of this paper. To Professor C. B. Rao, Presidency College, Madras, my thanks are due for granting permission to use the College Herbarium for identification of the host plants and to the University of Madras, for the award of a Studentship. I owe much to my father Mr. K. M. Thomas, Director of Research, Coffee Research Station, Balehonnur, who introduced me to the subject, for constant advice and for the facilities I obtained to carry out part of the investigations at Balehonnur. I also wish to express my thanks to all the planters who kindly gave ready answers to the questionnaire.

References

*Arens, K. .. "Untersuchungen über Keimung und Zytologie der Oosperon von *Plasmopara viticola* (Berl. et de Toni)," *Jahrb. wiss., Bot.*, 1929, **70**, 57–92.

*_____ .. "Die kutikulare Exkretion des Laubblattes," *Jahrb. wiss. Bot.*, 1934, **80**, 248–300.

*Bally, W. .. "Spinnewebziekten en djamoer oepas bij koffie," *Arch. Koffiecult.*, 1929, **3**, 1–26.

*Bavendamm, W. .. "Über das Vorkommen und den Nachweis von Oxydasen bei holzzerstorenden Pilzen," *Zeitschr. Pflanzenkrankh.*, 1928, **38**, 257–76.

Brian, P. W., Wright, J. M. Stubbs, J. and Way, A. M. "Uptake of antibiotic metabolites of soil micro-organisms by plants," *Nature*, 1951, **167**, 347.

Briton-Jones, H. R. and Baker, R. E. D. "Thread blights in Trinidad," *Tropical Agric.*, 1934, **11**, 55–67.

Burt, E. A. .. "Corticiums causing Pellicularia disease of the coffee plant, Hypochnose of pomaceous fruits and Rhizoctonia disease," *Ann. Mo. bot. Gard.*, 1918, **5**, 119–32.

Butler, E. J. .. *Fungi and Disease in Plants*, 1918, Thacker, Spink and Co., Calcutta and Simla.

Coleman, L. C., Venkata Rao, M. K. and Narasimhan, M. J. "Black rot or koleroga of coffee in Mysore," *Dep. Agric. Mysore State mycol. Ser. Bull.*, 1923, **5**, 1–12.

Cooke, M. C. .. "Some Indian fungi," *Grevillea*, 1876 *a*, **4**, 114–18.

——— .. "Affinities of *Pellicularia*," *Ibid.*, 1876 *b*, **4**, 134.

*De Bary, A. .. *Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria*," 1887, Oxford University Press, New York.

*Deslandes, J. A. .. "Observacœs fitopatologicos na Amazonia," *Bol. fitossan. Minist. Agric. Rio de J.*, 1944, **1**, 197–242.

*Ernst, A. .. "Botanische Notizen aus Caracas," *Bot. Zbl.*, 1880, 1178–79.

Fawcett, G. L. .. "*Pellicularia koleroga* on coffee in Puerto Rico," *J. agric. Res.*, 1914, **2**, 231–33.

Gäumann, E. .. *Principles of Plant Infection*, 1950, Crosby Lockwood and Son Ltd., London.

Hawker, L. E. .. *Physiology of Fungi*, 1950, University of London Press Ltd., London.

Higgins, B. B. .. "Physiology and parasitism of *Sclerotium rolfsii*," *Phytopathology*, 1927, **17**, 417–48.

*Höhnel, von F. .. "Fragmente zur Mykologie X. 470. *Corticium salmonicolor* B. and Br.," *Sitzb. k. akad. Wiss. Wien. Math-Natur. Wiss. klass.*, 1910, **119**, 395.

*Jackson, H. S. .. "Studies of Canadian Thelephoraceæ, II. Some new species of *Corticium*," *Canad. J. Res.* Sec. C., 1948, **26**, 143–57.

*Kuijper, J. .. "De Zilverdraadziekte der koffie in Suriname," *Dep. Landb. Suriname Bull.*, 1912, **28**, 11–24.

Kuttalalingam Pillay, M. A. .. *Eleventh Annual Report of the Indian Coffee Board, Bangalore* 1950–51, **1**, 1–218.

*Large, J. R. .. "Recent observations on thread blight *Corticium stevensii* of Tung and some native plants in the South-eastern U.S.A.," *Plant Dis. Reptr.*, 1943, **27**, 223–24.

*Marchionatta, J. B. .. "Una fitonosia nueva (La 'koleroga' del Cafe?)," *Physis Buenos Aires*, 1927, **8**, 554–57.

Mathew, K. T. .. "Growth factor requirements of *Pellicularia koleroga* Cooke in pure culture," *Nature*. 1952, **170**, 889–90.

Matsumoto, T. and Yamamoto, W. "*Hypochnus sasakii* Shirai in comparison with *Corticium stevensii* Burt and *Corticium koleroga* (Cooke) v. Höhn.," *Trans. nat. Hist. Soc. Formos.*, 1935, **25**, 161–75.

Mayne, W. W., Narasimhan, M. J. and Sreenivasan, K. H. "Spraying of coffee in South India," *Mysore Coffee Exp. Sta. Bull.*, 1933, **9**, 1–69.

Narasimhan, M. J. .. "Black rot of coffee in Mysore," *Phytopathology*, 1933, **23**, 875–86.

*Nowell, W. .. *Diseases of Crop Plants in the Lesser Antilles*," London W. Indies Committee, 1923.

Ramakrishnan, K. and Ramakrishnan, T. S. .. "Banded leaf blight of arrowroot, *Maranta arundinacea*," *Indian Phytopathology*, 1948, **1**, 129–36.

Ramanathan, V., Bal, D. V. and Thomas, K. M. "A review of the past experimental work on coffee in South India with suggestions for the future," *Indian Coffee Bd. Res. Dep.*, Balehonnur, Mysore, 1951.

Robbins, W. J. .. "Growth substances in agar," *Amer. J. Bot.*, 1939, **26**, 772–78.

Rogers, D. P. .. "The genus *Pellicularia* (Thelephoraceæ)," *Farlowia*, **1**, 95–118.

——— and Jackson, H. S. .. "Notes on the synonymy of some American Thelephoraceæ and other resupinates," *Ibid*, 1943, **1**, 263–328.

Stevens, F. L. and Hall, J. G. "Hypochnose of pomaceous fruits," *Ann. mycol.*, 1909, **7**, 49–59.

Thomas, K. M. .. "Second annual report of the Research Department of the Indian Coffee Board (1948–49)," *Bull. Res. Dep. Indian Coffee Bd.*, **2**, 1–54.

Tims, E. C. and Bonner, F. .. "Method of obtaining pure culture of *Corticium stevensii* from sclerotia," *Phytopathology*, 1942 *a*, **32**, 824–25.

*——— .. "Studies of fig leaf blights," *Proc. La. Acad. Sci.*, 1942 *b*, **6**, 13–34.

*Tucker, C. M. .. "Report of plant pathologist," *Rept. Porto Rico Agric. Exp. Sta.*, 1927, 24-40.

Tunstall, A. C. and Sarmah, K. C. "Black rot of tea in North-East India," *Tocklai Exp. Sta. Bull.*, 1940, **19**. 1–26.

*Venkatrayan, S. V. .. "Diseases of coffee," *Mysore Agric. J.*, 1947, **25**, 3–20.

——— .. "The validity of the name *Pellicularia koleroga* Cooke," *Indian Phytopathology*, 1949, **2**, 1–5.

*Weber, G. F. .. "Thread blight", a fungus disease of plants caused by *Corticium stevensii* Burt," *Florida Agric. Exp. Sta. Bull.*, 1927, **186**, 143–63.

——— .. "Web-blight, a disease of beans caused by *Corticium microsclerotia*", *Phytopathology*. 1939. **29**, 559–74.

.*Wellman, F. L., Walker, J. C. Cook, A. and Gallegly, M. "Primera Asamblea Latinoamericana de Fitoparasitologia," *Foll. misc. Ofic. Estud. esp. Mex.*, 1951, **4**, 251–57.

Wolf, F. A. .. "Further observations on *Corticium koleroga* (Cooke) v. Höhn., *Phytopathology*, 1928, **18**, 147–48.

———— and Bach, W. J... "The thread blight disease caused by *Corticium koleroga* (Cooke) v. Hohn. on citrus and pomaceous plants," *Phytopathology*, 1927, **17**, 689–709.

*Articles not seen in original.

EXPLANATION OF PLATE

FIG. 1. Shows a *Coffea arabica* plant severely defoliated through an attack of 'black rot' in July–August and 'rust' in December. Note the extent of die-back of the branches.

FIG. 2. Shows the characteristic hyphal thread of *Pellicularia koleroga* running along the shoot on to the leaf. The mycelial thread is slightly lifted on one side to show the connection to the leaf (× 3).

FIG. 3. Shows *C. arabica* berries in different stages of rotting. On the extreme left is a healthy berry. Note slight mummification in the diseased berries (× 1·5).

FIG. 4. Shows the fungus pellicle covering the lower surface of *C. arabica* leaves. Note the whitish, dense, powdery, spore-bearing patches (× 0·5).

1

2

3

4

PECTIC CHANGES IN PEARS DURING STORAGE AND RIPENING

By W. B. Date* and Elmer Hansen

(Department of Horticulture, Oregon State College, Corvallis, Oreg, U.S.A.)

Received July 17, 1953

(Communicated by Dr. C. S. Pichamuthu)

It is common practice in the Western countries to cold store apples and pears after their harvest either as a stop-gap arrangement or to develop certain quality in the fruit which cannot be obtained otherwise. In the course of the storage period, however, some changes take place in the fruit metabolism which have a bearing on the ripening of the fruit after taking them out from the storage. This is particularly so in the case of pears.

With the process of ripening in fruits are associated the changes in the pectic material of the fruit. Carre[2] made extensive studies of the changes in pectic material in apples during storage. She associated ripening in storage with an increase in the soluble pectin and a corresponding decrease in protopectin, the total pectic material remaining constant. She has also presented some evidence to show that the total pectin content tends to increase as apples ripen. These pectic changes are, however, related to the temperature of holding the fruit. Echevin[5] has shown that in pears pectic changes are directly proportional to the temperature of storage. Lutz and Culpepper[13] found a direct correlation between optimum ripening and maximum soluble pectin formation in Keiffer pears at 60° F. Smock, Gerhardt and Plagge[15, 7, 14] have also correlated pectic substances with the storage behaviour of Bartlett pears in artificial atmospheres containing carbon-dioxide atmospheres. Gerhardt[6] has shown the effect of filtration of ethylene from cold storage on some of the physiological changes in Bartlett and Anjou pears.

Thus normal ripening is associated with progressive changes in the physical texture of the fruit, which passes from firmness and crispness to a soft, smooth and buttery consistency. This change is directly associated with the hydrolysis of the insoluble protopectin into soluble pectin and pectic acid. As a part of the cold storage investigations on pears at the Department of Horticulture a study of the chemical changes in fruits was enlarged to include pectic changes in pears.

* Present Address—Central Food Technological Research Institute, Mysore.

METHODS AND MATERIALS

Three varieties of pears, Bartlett, Bosc and Anjou were obtained from the Hood River experiment station orchard. The fruits were packed with oil paper wraps. Sufficient fruit was put into the cold rooms to provide monthly samples during the normal storage period of the variety. One of the rooms was equipped with a Dorex unit cannister having a capacity of 35 c.f.m. providing 6 complete recirculations of air in the room per hour. The makers of this unit claimed that it filters out ethylene from storage atmosphere. The fruit samples under these conditions are hereafter referred to as the carbon treated samples (C.T.). Samples from the room without the air purifying unit are referred to as control samples (C.).

The temperature in each room was maintained at 30°–31° F. throughout the storage period.

At monthly intervals, a 60 fruit sample from each storage treatment was placed in a ripening room maintained at 68–70° F. At intervals of 0, 3, 6, 9, 12 days, 12 pears were sampled for chemical analysis.

A 250 gm. aliquot of the ground material representing all the twelve pears was transferred to sufficient hot 95% isopropyl alcohol to give a final concentration of 80% alcohol and boiled for 15 minutes. After cooling the alcohol insoluble material was separated by filtering, washed 3–4 times with 80% alcohol and then was transferred to a beaker and dried at 45° C. Pectin fractions were estimated using this material by employing the method of Carre.[3] The results of analysis are presented in the Tables I to III.

EXPERIMENTAL RESULTS AND DISCUSSION

Changes during storage.—As shown in the tables the amount of both protopectin and total pectin increased during the period of storage in all the varieties. In Anjous and Bartletts, the initial increase was followed by a decline towards the latter part of the storage period. According to these data it might be assumed that an actual synthesis of protopectin continues to occur after the fruits are harvested and placed in storage. Anjou pears contained more total and protopectin than either Bartlett or Bosc at all stages of storage. The initial soluble pectin content in all the varieties was higher than those reported by Gerhardt and Ezell[7] for similar varieties grown in other regions.

The use of activated carbon appears to have had no consistent effect on retarding the rate of pectic changes in any variety during storage. In case of early varieties like Bartlett and Bosc, there are appreciable differences in pectin and total pectin content over longer periods. In Anjous, a late

TABLE I

Pectic Changes in Bartlett Pears during Storage and Ripening

(Pectic material weighed as calcium–pectate and expressed as per cent. of fresh weight).

Months	No. of days ripened	Control			Carbon treated		
		Pectin	Proto-pectin	Total pectin	Pectin	Proto-pectin	Total pectin
October ..	0	0·3070	0·3765	0·6835	0·2158	0·5294	0·7452
	3	0·3028	0·3554	0·6582	0·3200	0·3914	0·7114
	6	0·3683	0·3739	0·7422	0·3323	0·2751	0·7074
	9	0·2813	0·3683	0·6496	0·2458	0·2741	0·5199
	12	0·1498	0·1583	0·3081	0·1571	0·1124	0·2695
November ..	0	0·2018	0·5091	0·7109	0·2098	0·4897	0·6990
	3	0·3819	0·4483	0·8301	0·4260	0·4400	0·8660
	6	0·2997	0·4666	0·7664	0·3475	0·4051	0·7527
	9	0·2132	0·3586	0·5719	0·2287	0·3588	0·5876
	12	0·1198	0·2650	0·3848	0·1368	0·2695	0·4063
December ..	0	0·2374	0·6513	0·8887	0·2816	0·6204	0·9019
	3	0·3522	0·5020	0·8543	0·3545	0·4377	0·7922
	6	0·2349	0·5149	0·7498	0·2401	0·4686	0·7088
	9	0·1185	0·3634	0·4813	0·1274	0·4141	0·5510
	12	0·1527	0·3044	0·4572	0·1334	0·3240	0·4574
January ..	0	0·2574	0·4945	0·7518	0·3102	0·4844	0·7946
	3	0·1691	0·6579	0·8270	0·2963	0·6214	0·9177
	6	0·1624	0·5307	0·6932	0·1964	0·5182	0·7146
	9	0·1632	0·4289	0·5921	0·1698	0·4105	0·5803
	12	0·1630	0·4587	0·6217	0·1905	0·4456	0·6361
February ..	0	0·2157	0·4808	0·6965	0·1588	0·4442	0·6030
	6	0·1487	0·4223	0·5710	0·1698	0·3678	0·5376

variety, fruit from carbon treated room contained, in February and March, more protopectin and less soluble pectin indicating that the rate of hydrolysis had been retarded by the use of activated charcoal as a means of storage air purification.

Changes during ripening.—Although considerable variations were found relative to the change in the total pectin content during ripening certain trends are apparent. In all the varieties there was a tendency for the total pectin content to increase generally during the early stages of ripening. This behaviour was especially apparent in Anjou pears, but was also observed in certain samples of Bartlett and Bosc. In most cases the initial increase in total pectin was followed by a decline, so that at the end of twelve days there was an actual decrease below the amount originally present.

TABLE II

Pectic Changes in Bosc Pears during Storage and Ripening

(Pectic material weighed as calcium-pectate and expressed as per cent. of fresh weight)

Months	No. of days ripened	Control			Carban treated		
		Pectin	Proto-pectin	Total pectin	Pectin	Proto-pectin	Total pectin
October ..	0	0·2072	0·3805	0·5877	0·2093	0·3729	0·5822
	3	0·1875	0·4870	0·6745	0·1577	0·4371	0·5948
	6	0·3124	0·2879	0·6003	0·3323	0·2560	0·5883
	9	0·3553	0·2500	0·6053	0·3644	0·2738	0·6382
	12	0·2477	0·1689	0·4166	0·2456	0·1873	0·4329
November ..	0	0·2912	0·3990	0·6902	0·2483	0·4047	0·6530
	3	0·1899	0·3387	0·5287	0·2192	0·3539	0·5732
	6	0·2715	0·3359	0·6074	0·2811	0·3135	0·5947
	9	0·2485	0·2397	0·4882	0·2565	0·2488	0·5055
	12	0·2715	0·3463	0·6178	0·3281	0·3155	0·6435
December ..	0	0·2208	0·4796	0·7005	0·2254	0·4320	0·6574
	3	0·1911	0·4982	0·6894	0·2124	0·4952	0·7076
	6	0·3614	0·4197	0·7811	0·3759	0·3836	0·7595
	9	0·3341	0·3870	0·7211	0·3409	0·3752	0·7160
	12	0·1880	0·2539	0·4419	0·2203	0·3112	0·5315
January ..	0	0·1567	0·5593	0·7160	0·1931	0·5545	0·7476
	3	0·2562	0·5178	0·7739	0·2217	0·5059	0·7276
	6	0·2522	0·3997	0·6519	0·2927	0·4583	0·7510
	9	0·2944	0·3156	0·6099	0·3201	0·3197	0·6398
	12	0·2324	0·2506	0·4830	0·2601	0·3224	0·5825
February ..	0	0·2224	0·5434	0·7658	0·2187	0·5189	0·7376
	3	0·1961	0·4154	0·6115	0·1992	0·3864	0·5855
	6	0·3450	0·4113	0·7562	0·3439	0·4767	0·8206
	9	0·2765	0·3795	0·6559	0·2365	0·4254	0·6619
	12	0·2709	0·3576	0·6285	0·2197	0·3054	0·5251

The amount of change which occurred in the protopectin fraction during ripening was different in the samples analysed after progressive periods of storage. In Bartlett and Bosc, protopectin decreased during ripening. The ability of the tissues to hydrolyse this material became progressively less, the longer the fruit remained in the storage. Owing to the increase in protopectin content which occurred during storage period, Bartlett pears, in January, even after being kept for 12 days in the ripening room, still contained more protopectin than was originally present in the unripened fruit in October. Bosc pears behaved similarly. Anjou pears transferred to the ripening room in October showed no reduction in protopectin content at the end of 12 days. Samples ripened at later dates, however, showed

TABLE III

Pectic Changes in Anjou Pears during Storage and Ripening

(Pectic material weighed as calcium–pectate and exprcssed as per cent. of fresh weight)

Months	No. of days ripened	Control			Carbon treated		
		Pectin	Proto-pectin	Total pectin	Pectin	Proto-pectin	Total pectin
October ..	0	0·2955	0·4732	0·7687	0·1687	0·5280	0·6967
	3	0·1782	0·4733	0·6515	0·1764	0·5266	0·7030
	6	0·1590	0·6286	0·7876	0·1479	0·5017	0·6496
	9	0·1887	0·4513	0·6400	0·1520	0·4880	0·6400
	12	0·2610	0·5245	0·7855	0·3118	0·3895	0·7013
November ..	0	0·2016	0·4307	0·6323	0·2540	0·4688	0·7228
	3	0·2148	0·5697	0·7844	0·1736	0·5417	0·7153
	6	0·3488	0·4241	0·7729	0·1792	0·5094	0·6886
	9	0·5739	0·3799	0·9538	0·4232	0·4637	0·8867
	12	0·5219	0·3106	0·8325	0·4992	0·3674	0·8666
December ..	0	0·1715	0·6897	0·8613	0·1835	0·6460	0·8295
	3	0·2715	0·6250	0·8967	0·2265	0·6739	0·9004
	6	0·4615	0·4448	0·9063	0·4348	0·4612	0·8960
	9	0·5863	0·3896	0·9759	0·5820	0·3334	0·9154
	12	0·4208	0·3034	0·7242	0·4252	0·2973	0·7225
January ..	0	0·1905	0·7423	0·9328	0·1807	0·6774	0·8581
	3	0·2278	0·6292	0·8570	0·2289	0·5775	0·8073
	6	0·4688	0·4660	0·9348	0·4907	0·4637	0·9544
	9	0·5868	0·3314	0·9182	0·6531	0·3211	0·8842
	12	0·6836	0·3823	1·0659	0·6825	0·3932	1·0757
February ..	0	0·2320	0·4730	0·7050	0·1177	0·6298	0·7475
	3	0·4268	0·5169	0·9437	0·2996	0·6992	0·9888
	6	0·5600	0·4057	0·9657	0·5822	0·4069	0·9891
	9	0·5694	0·3517	0·9211	0·5539	0·3519	0·9058
	12	0·3694	0·3289	0·6983	0·5003	0·3040	0·8043
March ..	0	0·3607	0·4484	0·8091	0·2076	0·5687	0·7763
	3	0·3894	0·4280	0·8174	0·4030	0·6349	1·0379
	6	0·4034	0·3795	0·7829	0·5560	0·3834	0·9394
	9	0·3181	0·3018	0·6198	0·4180	0·3455	0·7635
	12	0·3386	0·4182	0·7568	0·3625	0·3246	0·6870

rapid, consistent decreases, resulting in most of the protopectin being hydrolysed by the end of 12 days.

The changes in soluble pectin during ripening in the three varieties were different in several respects. In Bartlett pears, with the exception of the January sample, pectin accumulated for three to six days and then declined rapidly. In Bosc, the increase in pectin content occurred over a longer period of time and declined to less extent in the post-ripening period. Anjou

fruit being in a preclimacteric period showed no increases in soluble pectin in October. Samples ripened in December and at monthly intervals thereafter showed consistent increases in soluble pectin.

The accumulation of soluble pectin which occurred at ripening temperatures could not in all cases be accounted for by the concomittant decrease in protopectin. It would appear from the results that the soluble pectin which accumulated during ripening was not always derived exclusively from the hydrolysis of protopectin.

The use of activated carbon during the storage period appears to have resulted in no appreciable effects on the pectic changes during ripening. While differences in the rate and magnitude were found in some cases, no consistent trends are apparent.

The question arises as to what changes occur in the metabolism of the pectic substances during prolonged low temperature storage which result in a decline or loss in the ability of the fruit to soften during the ripening process. Gerhardt and Ezell[7] found no evidence to indicate that the apparent inactivation resulted directly from low temperature injury, as suggested by Kidd and West.[11, 12] In fact they[7] found that its inception was earlier and more severe at 36° F. than at 30° F. They conclude that the loss in ripening capacity in pears can be explained by the inactivation of protopectinase system during the cold storage period. Kertesz[10] and Griffen and Kertesz[8] however, were unable to show the presence in fruits of an enzyme which hydrolyzes protopectin. According to them this is brought about by organic acids.

The data obtained in this investigation show that during storage the protopectin content actually increased and it would appear that the synthesis of these substances continues after the fruit has become mature and is removed from the tree. Heinze and Appleman[9] have indicated that there is a similar increase in the protopectin content of sweet potatoes during storage.

Arreguin and Bonner[1] have shown that in the interchanges between starch and sugars in potatoes phosphorylation reactions are mainly responsible. Study in that light was not done in case of pears but it is possible that according to the evidence presented by various authors[4] the reactions leading to a gain or loss in pectic substances would be more understandable in the light of this newer knowledge of plant metabolism.

SUMMARY

A study has been made of the pectic changes in different varieties of pears under cold storage and periodical ripening as a part of the different

chemical changes taking place in the fruit under such conditions. The nature of pectic changes could be summarised as follows:

1. The amounts of both protopectin and total pectin increased during storage in all the three varieties.

2. Anjou pears contained more total and protopectin than the other two varieties.

3. The data indicate a possibility of the synthesis of protopectin during storage.

Literature Cited

1. Arreguin, B. and Bonner, J. "Experiments on sucrose formation by potato tubers as influenced by temperature," *Plant Physiology*, 1949, **27**, 720–38.
2. Carre, M. H. .. "The pectic constituents of stored fruits," *The Biochemical Journal*, 1922, **16**, 704–12.
3. ——— and Haynes, D. "The estimation of pectin as calcium pectate and the application of this method to the determination of soluble pectin in apples," *Ibid.*, 1922, **16**, 60–69.
4. Cardini, C. E., Paladini, A. C., Caputo, R. and Leloir, L. E. "Uridine diphosphate glucose: the co-enzyme of the glactose-glucose phosphate isomerization," *Nature*, 1950, **165**, 191–92.
5. Echevin, R. .. "L'evolution du complexe pecto-cellulosique dans les fruits conserves par le froid," *Comptes Rendus*, 1941, **213** (14), 458–60.
6. Gerhardt, F. .. "Air purification in apple and pear storages," *Refrigerating Engineering*, 1950, **58**, 145–48, 192–94.
7. ——— and Ezell, B. D. "Physiological investigations on Fall and Winter Pears in Pacific Northwest," *U.S.D.A. Technical Bulletin*, 1941, No. 759, 66.
8. Griffen, J. H. and Kertesz, Z. I. "Changes which occur in apple tissue upon treatment with various agents and their relation to the natural mechanism of softening during maturation". *The Botanical Gazette*, 1946–47, **108**, 279–85.
9. Heinze, P. H. and Appleman, C. O. "A biochemical study of curing processes in sweet potatoes," *Plant Physiology*, 1943, **18**, 548–55.
10. Kertesz, Z. I. .. "A possible non-enzymic mechanism of changes occurring in the pectic substances and other polysaccharides in living plants," *Ibid.*, 1943, **18**, 308–09.
11. Kidd, F. and West, C. .. "The gas storage of pears (Great Britain)," *D.S.I.R. Food Investigation Board Report for* 1931, 1932, 92–99.
12. ——— .. "Low temperature injury in the cool storage of fruits and vegetables (Great Britain)," *Ibid.*, 1932, 122–27.

13. Lutz, J. M. and Culpepper, C. W. "Certain chemical and physical changes produced in Keiffer pears during ripening and storage," *U.S.D.A. Technical Bulletin*, 1937, No. 590, 37.

14. Plagge, H. H. and Fisher, D. V. "Pectic changes in Jonathan apples as a measure of ripening under differential carbon-dioxide treatments," *American Society of Horticultural Science Proceedings*, 1942, **40**, 169–71.

15. Smock, R. M. and Allen, F. W. "Soluble pectin changes in gas stored fruits," *Ibid.*, 1937, **35**, 184–87.

)26–54 Printed at The Bangalore Press, Bangalore City, by C. Vasudeva Rao, Superintendent
and Published by The Indian Academy of Sciences, Bangalore

STUDIES ON THE BLACK ROT OF COFFEE

II. Nutritional Requirements of *Pellicularia koleroga* Cooke with Special Reference to Growth Substances*

By K. T. Mathew

(*University Botany Laboratory, Madras*)

Received May 5, 1953

(Communicated by Professor T. S. Sadasivan, M.Sc., Ph.D., F.A.Sc.)

In an earlier note certain observations on the growth substance requirements of *Pellicularia koleroga* Cooke [= *Corticium koleroga* (Cooke) v. Höhnel] in pure culture was briefly reported (Mathew, 1952). This contribution is the outcome of further work on similar lines and in this larger paper the previous results are also included and dealt in greater detail.

Materials and Methods

Isolates 1, 2, 3, 4, 5, 7, 10 and 11 used in these investigations were all single hyphal tip cultures of the fungus *P. koleroga* isolated by the author from different hosts. The method of isolation, maintenance and other details of the different isolates were described earlier (Mathew, 1954). Isolate 12 was a pure culture of *Corticium koleroga* (Cooke) v. Höhnel, obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

All general precautions essential in growth substance studies were taken. The glassware used was Pyrex, distilled water was obtained from an all-glass Pyrex still, the chemicals were of the highest purity and the details of these and the methods employed in the purification of agar, preparation of media and in recording the growth of the fungus were all the same as described in a previous paper (Mathew, 1953).

Two media I *a* and I *b* were very frequently used and medium I *a*, had the following composition: KNO_3 2 g., KH_2PO_4 1 g., $MgSO_4 \cdot 7H_2O$ 0·5 g., glucose 20 g. and water to make 1000 ml. The same solution solidified with 2% purified agar is referred as medium I *b*. The composition of all the other media will be given with the respective experiments. These media were not essentially free of the commoner trace elements necessary in fungal nutrition and hence further additions were thought unnecessary (Mathew, 1953). Unless otherwise stated the pH of the various media employed varied between 5 to 6 at the time of inoculation. In all experiments flasks

*Part of Thesis approved for the Degree of Doctor of Philosophy of the University of Madras.

used were of 250 ml. capacity with 20 ml. of solution, while in test-tubes 5 ml. of solid medium were used. The growth substances were added before autoclaving and the media sterilised at 10 pounds pressure for 15 minutes.

For inoculation, small bits of ærial mycelium removed from potato sucrose agar slants were used, care being taken not to touch the agar. Later, when the ærial mycelium produced was found scanty, the fungus was grown for 2 to 3 weeks in medium I *a*, supplemented with 2 μg. of thiamine per flask, the medium drained off, the fungus washed with sterile glass distilled water and small bits of mycelium removed with a needle and used for inoculation.

The cultures were always incubated in the dark at temperatures varying from 21 to 23° C. In all experiments the mean values given are that of not less than three replicates and the standard error of the mean was also calculated.

EXPERIMENTAL

Influence of temperature on growth of Pellicularia koleroga (*isolate* 1)

Into each 10 cm. Petri dish 10 ml. potato sucrose agar were poured and these were inoculated in the centre with uniform discs (3 mm.) cut with a cork borer from actively growing fungal colonies. The average of the maximum and minimum diameter for each colony was recorded.

In all future work the fungus was incubated at 21 to 23° C. which was optimum for growth (Table I) and the result was in agreement with that of

TABLE I

Influence of temperature on radial growth of Pellicularia koleroga (*isolate* 1) *in potato sucrose agar*

Temperature	Growth of fungus in cm.			
	2 days	4 days	6 days	7 days
7– 8° C. ..	0·1±0·0	0·6±0·0	1·2±0·0	1·7±0·2
16–18° C. ..	0·9±0·2	2·3±0·2	4·0±0·1	5·0±0·4
21–23° C. ..	1·3±0·0	3·2±0·2	4·7±0·4	6·0±0·4
27–28° C. ..	0·9±0·0	1·2±0·2	2·2±0·4	2·5±0·4
31–32° C. ..	0·0±0·0	0·0±0·0	0·0±0·0	0·0±0·0

Matsumoto and Yamamoto (1935). At the low temperature of 7 to 8° C. the fungus made only a feeble growth. When the dishes were incubated at 31 to 32° C. there was no growth, but on transferring the same dishes to 21 to 23° C. after a week, the fungus readily grew.

Growth of Pellicularia koleroga (*isolate* 1) *in different media*

The fungus was cultured on the following solid media in test-tubes: Barnes agar, Richard's agar, potato sucrose agar, carrot agar, bean agar, malt agar, onion agar, banana agar, oat agar, rice agar, coffee leaf extract agar and potato cylinder.

On Barnes and Richard's agar the fungus made only very poor growth. All the natural media supported excellent growth of the fungus and on all these except banana agar, numerous minute, flattened, subspherical sclerotia were formed, both on the surface of the medium and on the surface of the glass, where the ærial mycelium had spread, after 10 days growth. The sclerotia were 3 to 4 times larger than those found in nature and measured 210 to 1,400 μ in diameter. On banana agar and coffee leaf extract agar media a brown ring or halo was formed in advance of the growing mycelium like that observed by Narasimhan (1933) on medium to which tannic acid was added and this is thought to be due to the tannin present in banana and coffee leaves. The failure of the fungus to grow in synthetic media suggested some growth substance deficiency and this aspect of its nutrition received importance in all future work. A certain amount of attention was also paid to the carbon and nitrogen requirements of the fungus.

Effect of addition of extracts of natural products, fungal mats and staled fungal medium to medium I *b on growth of* Pellicularia koleroga (*isolate* 1)

Small quantities (0·05 to 0·1 ml.) of dilute extracts of lentil leaves, coffee leaves, malt extract, yeast powder and fungal mat extracts of *Fusarium vasinfectum*, *F. moniliforme*, *Rhizoctonia bataticola* and *Penicillium notatum* were added to 5 ml. each of medium I *b* in test-tubes. Similar amounts of the staled fungal medium I *a* in which the above fungi were grown for a week, were also added to other tubes.

After 21 days the growth of the fungus was negligible in the controls to which no additions were made, while in all the other tubes the fungus grew well. This confirmed that the fungus was heterotrophic for one or more growth substances and that these could be supplied by many natural products as well as other autotrophic fungi. That the natural products mentioned are good sources of growth substances and that autotrophic fungi synthesise their own requirements of growth substances are established

facts (Hawker, 1936, 1939 *a*, 1948; Asthana and Hawker, 1936; Kögl and Fries, 1937; Müller and Schopfer, 1937; Robbins and Kavanagh, 1938 *a*; Leonian and Lilly, 1940; Robbins and Ma, 1942; Schopfer, 1943; Fries, 1948). Since very small quantities of the dilute extracts produced a marked effect on the growth of the fungus, it was assumed that the growth substances essential were of the nature of a vitamin.

Results of some preliminary experiments on the effect of growth substances on the growth of Pellicularia koleroga (*isolate* 1)

A start was made with a few vitamins obtained in ampoules from a drug store. Thiamine and biotin were added individually at the rate of 2 μg. to flasks containing 20 ml. each of Richard's synthetic solution. The controls without the vitamins showed only a negligible amount of growth of the fungus, while good growth was observed in the presence of both thiamine and biotin at the end of 15 days.

When a few more vitamins were available, they were also tested. Medium I *a* was distributed into flasks and the following additions were made: none, thiamine 5 μg., riboflavine 5 μg., biotin 2 μg., inositol 2 mg., thiamine + riboflavine + biotin, thiamine + riboflavine + inositol, thiamine + biotin + inositol, riboflavine + biotin + inositol, thiamine + riboflavine + biotin + inositol and malt extract 20 mg.

The cultures were visually compared after 15 days. The growth of the fungus was negligible in the controls and in the *plus* inositol series. Thiamine, riboflavine and biotin each singly promoted good growth of the fungus; growth with thiamine was superior to that of the other two and almost equal to the growth obtained with all the four vitamins in combination. The exclusion of any single vitamin from the mixture of four, did not affect the growth to any appreciable extent. The best growth was seen in the malt extract added flasks.

Pure crystalline growth substances were available after a period of abou 6 months and they were used in all subsequent experiments. The following additions were made per tube to medium I *b*: none, thiamine 5 μg., riboflavine 5 μg., pyridoxine 5 μg., pantothenic acid 5 μg., nicotinic acid 5 μg., biotin 2 μg., inositol 2 mg. and *p*-aminobenzoic acid 5 μg. At the end of 30 days only thiamine and riboflavine had promoted growth of the fungus and among these growth was much faster with thiamine.

Since in earlier trials good growth was obtained with biotin also, the experiment was repeated. However, the amount of thiamine, riboflavine, pyridoxine, pantothenic acid, nicotinic acid and *p*-aminobenzoic acid was

each reduced to 1 μg. per tube, that of biotin to 0·1 μg. and inositol to 0·1 mg. respectively. When observed at the end of 30 days only thiamine supplied tubes had supported growth of the fungus.

Influence of glucose and sucrose on response of Pellicularia koleroga (*isolate* 1) *to growth substances*

The varying results reported above needed further investigation and to further minimise the chances of errors, the experiment was conducted in liquid cultures on media containing both glucose and sucrose as carbon sources. Medium I *a* was used in one set of flasks and in the other set the glucose in the medium was replaced by an equal amount of sucrose. The various treatments were as follows: none, thiamine 5 μg., riboflavine 5 μg., pyridoxine 5 μg., pantothenic acid 5 μg., nicotinic acid 5 μg., biotin 2 μg., inositol 2 mg. and *p*-aminobenzoic acid 5 μg.

The interesting observation that only thiamine could promote growth when glucose was the carbon source, while both thiamine and biotin individually had the ability to do so when sucrose replaced glucose (Table II) was published in the earlier note (Mathew, 1952).

TABLE II

Effect of addition of growth substances to medium I a, with glucose and sucrose as carbon sources, on growth of Pellicularia koleroga (*isolate* 1)

Growth substance	Growth ratings				Dry wt. of fungus in mg.	
	7 days		15 days		30 days	
	Glucose	Sucrose	Glucose	Sucrose	Glucose	Sucrose
None	*n*	*n*	*n*	*n*	*n*	*n*
Thiamine 5 μg. ..	*	*	***	**	129·5 ± 12·8	89·9 ± 4·8
Riboflavine 5 μg. ..	*n*	*n*	*n*	*n*	*n*	*n*
Pyridoxine 5 μg. ..	*n*	*n*	*n*	*n*	*n*	*n*
Pantothenic acid 5 μg.	*n*	*n*	*n*	*n*	*n*	*n*
Nicotinic acid 5 μg. ..	*n*	*n*	*n*	*n*	*n*	*n*
Biotin 2 μg. ..	*n*	*n*	*n*	*	*n*	59·7 ± 5·1
Inositol 2 mg. ..	*n*	*n*	*n*	*n*	*n*	*n*
p-aminobenzoic acid 5 μg.	*n*	*n*	*n*	*n*	*n*	*n*

n: negligible; * poor; ** fair; *** good.

Influence of pH on growth of Pellicularia koleroga *(isolate* 1)

The purpose of this experiment was to determine a pH range suitable for the growth of the fungus in future work and also to see whether small differences in pH caused by addition of growth substances in the various treatments would affect its growth to any marked extent. Since many of the growth substances while stable in an acidic medium are destroyed if autoclaved in an alkaline solution, only growth at pH levels varying from 2·5 to 7·3 were investigated. The different pH levels were adjusted by adding required amounts of either H_2SO_4 or NaOH of known strength and recorded with the help of Lovibond comparator discs and B.D.H. indicators. To each flask containing 20 ml. of medium I *a*, thiamine was added at the rate of 2 μg. per flask.

TABLE III

Influence of pH on growth of Pellicularia koleroga *(isolate* 1), *in medium I a*

Initial pH	Growth ratings		Dry wt. of fungus in mg.	Final pH
	7 days	15 days	21 days	
2·5	0	0	0·0±0·0	..
3·8	**	***	92·8±2·9	7·8
4·3	**	***	96·7±4·4	7·8
5·4	**	***	95·9±8·1	7·8
6·3	**	***	94·0±1·4	8·0
7·3	**	***	107·0±3·6	8·0

0 : nil; ** fair; *** good.

Although maximum growth was recorded in a slightly alkaline solution at pH 7·3 (Table III), for all future work also the pH range between 5 to 6 which was used so far, was preferred for two reasons: (1) it was more convenient to make additions of growth substances before autoclaving and for this an acidic range was necessary and (2) the growth of the fungus was not materially affected by changes in pH from 4 to 6. The final pH recorded after 21 days growth was found to be between 7·8 to 8·0 in all treatments irrespective of the initial pH.

Effect of exclusion of a single growth substance from a mixture ' M ' on growth of Pellicularia koleroga *(isolate* 1) *in medium I a*

To investigate whether the fungus was completely thiamine heterotrophic when cultured on medium I *a* and also to test whether it had partial deficiencies for any of the other growth substances a vitamin mixture ' M ' was composed as follows: thiamine, riboflavine, pyridoxine, pantothenic acid, nicotinic acid, *p*-aminobenzoic acid each 2 μg., biotin 0·5 μg. and inositol 1 mg.; this formed one treatment. In each of the other treatments a single growth substance was excluded from the mixture. In another treatment malt extract was added at the rate of 1 g. per litre instead of the mixture of vitamins.

When thiamine was withdrawn from the mixture, although the growth of the fungus was considerably reduced, it was superior to the controls (Table IV), and this showed that the growth substances other than thiamine in combination could still promote some growth of the fungus, although individually they would not (Table II). However, in media containing thiamine the fungus showed no partial deficiency for any of the other growth substances and the exclusion of any one of them did not significantly reduce its rate or amount of growth.

TABLE IV

Effect of exclusion of a single growth substance from a mixture ' M ' on growth of Pellicularia koleroga *(isolate* 1), *in medium I a*

Growth substance	Growth ratings		Dry wt. of fungus in mg.
	7 days	15 days	30 days
None	*n*	*n*	3·6±0·6
Malt extract	*	*(*)	58·3±7·0
Mixture 'M'	*	**	91·6±5·3
'M'-thiamine	*n*	*	17·4±1·3
'M'-riboflavine	*	**	92·8±2·0
'M'-pyridoxine	*	**	87·8±5·3
'M'-pantothenic acid	*	**	82·3±5·6
'M'-nicotinic acid	*	**	90·3±5·5
'M'-biotin	*	**	81·5±6·1
'M'-inositol	*	**	81·7±5·3
'M'-*p*-aminobenzoic acid ..	*	**	83·7±8·6

Mixture 'M': thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, *p*-aminobenzoic acid each 2 μg., biotin 0·5 μg. and inositol 1 mg.

n: negligible; * poor; ** fair; ( ) : denotes less than.

Growth of Pellicularia koleroga (*isolate* 1), *with different sources of carbon*

It was observed that the growth substance requirements of the fungus varied with a change in carbon source (Table II). Deficiencies for growth substances conditioned by the concentration of the medium (Robbins and Kavanagh, 1938 *b*; Saksena, 1941), carbon source (Fromageot and Tschang, 1938), nitrogen source (Stokes, Foster and Woodward, 1943), pH of medium (Lilly and Barnett, 1947) and temperature (Mitchell and Houlahan, 1946; Barnett and Lilly, 1948) have been recorded. Similarly it was possible that there was some cultural condition in which this fungus would also grow without the addition of any growth substances. The purpose of the experiment was also to test the ability of *P. koleroga* to assimilate the different sources of carbon. Numerous investigators have shown that carbohydrates are generally the best sources of carbon for most fungi. Among these glucose and fructose are almost universally used, though they do not necessarily give the best growth, while different fungi show varying abilities to use the other hexoses, pentoses, disaccharides, trisaccharides and polysaccharides (Hawkins, 1915; Brannon, 1923; Farries and Bell, 1930; Horr, 1936; Mosher, Saunders, Kingsley and Williams, 1936; Steinberg, 1939; Hawker, 1939 *b*, 1947 *a*; Studt, 1941; Ramakrishnan, 1941; Corum, 1942; Hawker and Chaudhri, 1946; Leben and Keitt, 1948; Cantino, 1948; Norkrans, 1950). Other sources of carbon like organic acids, higher alcohols and amino acids are relatively poorly used by most fungi (Mosher *et al.*, 1936; Studt, 1941; Leben and Keitt, 1948; Cantino, 1948). An exception is *Leptomitus lacteus* which is unable to assimilate the common sugars, but uses acetate, pyruvate, lactate and also fatty acids (Schade, 1940).

The glucose in medium I *b* was replaced by equal amounts of (2%) different sources of carbon. As some of the carbon sources were not of the highest purity and might have contained minute amounts of various growth substances as impurities, to get more uniform results, malt extract was used as the source of growth substances. One series of tubes without the addition of malt were kept as controls and to the other malt was added at the rate of 200 mg. per litre.

After 30 days, growth of the fungus was negligible in all the tubes from which malt was omitted irrespective of the carbon source. Among the carbon sources tried maltose, starch, galactose and mannose supported the best growth of the fungus in the presence of malt. These were followed by glucose, sucrose and glycerol. With mannitol the fungus made a fair amount of growth, but with xylose and lactose the growth was poor. Dulcitol permitted no growth of the fungus.

Inability to synthesise the necessary hydrolytic enzymes seems to be the most common cause for non-utilisation of some of the complex carbohydrates (Lilly and Barnett, 1951). Since glucose and galactose are readily used by the fungus, its failure to grow well on lactose should be due to its inability to hydrolyse it.

The experiment was repeated in liquid cultures with slight modifications, to determine the best carbon source for the growth of the fungus, with a few of the sugars which supported good growth. Medium I *a* was used as standard and in the different treatments the glucose in the medium was replaced by equal amounts of the other carbon sources as before. Instead of malt extract a mixture of thiamine 2 μg., riboflavine 2 μg. and biotin 0·1 μg. were added to each flask.

TABLE V

Growth of Pellicularia koleroga (*isolate* 1) *on medium I a, with different sources of carbon replacing glucose*

Carbon source	Growth ratings		Dry wt. of fungus in mg.
	7 days	15 days	21 days
None	*n*	*n*	1·7±0·4
Glucose	*	**(*)	91·6±1·4
Mannose	*	**(*)	115·7±1·6
Galactose	*	**(*)	88·3±2·7
Maltose	*	***	135·2±5·7
Sucrose	(*)	*(*)	61·3±2·1

n: negligible; * poor; ** fair; *** good; ( ): denotes less than.

The highest yield of the fungus was recorded when maltose was the carbon source, followed by mannose. Glucose and galactose were equally good, sucrose yielding the lowest weight (Table V).

Growth of Pellicularia koleroga (*isolate* 1), *with different sources of nitrogen*

The influence of various sources of nitrogen on the growth of the fungus was investigated on similar lines and for the same reasons mentioned under

the experiments with carbon sources. Robbins (1937) classified fungi into four groups based on their nitrogen requirements.

Group I .. Fungi able to use atmospheric, nitrate, ammonium and organic nitrogen. There is very little agreement on ability of fungi to fix atmospheric nitrogen (Lilly and Barnett, 1951).

Group II .. Fungi able to use only nitrate, ammonium and organic nitrogen, *e.g.*, *Aspergillus niger* (Steinberg, 1937), *Colletotrichum falcatum* (Ramakrishnan, 1941), *Venturia inæqualis* (Leben and Keitt, 1948).

Group III .. Fungi utilising only ammonium and organic nitrogen, *e.g.*, *Phycomyces blakesleeanus* (Schopfer, 1934).

Group IV .. Fungi utilising only organic nitrogen, *e.g.*, *Nematospora gossypii* (Farries and Bell, 1930), *Trichophyton interdigitale* (Mosher *et al.*, 1936), *Leptomitus lacteus* (Schade, 1940), *Blastocladia pringsheimii* (Cantino, 1948).

Medium I *b* was taken as standard and calculated quantities of the different sources of nitrogen were added in the other treatments, so as to give the same amount of nitrogen present in 2 g. of KNO_3. Nitrate, ammonium and organic forms of nitrogen were tried. The fungus was cultured in test-tubes with 200 mg. of malt extract per litre supplying the growth substances.

When no malt was added to the medium, the growth of the fungus was negligible with all the nitrogen sources. In the malt added series the best growth was obtained with KNO_3 and asparagine. Ammonium tartrate and urea were slightly inferior, followed by ammonium sulphate. According to the classification of Robbins (1937) *P. koleroga* is a group II fungus.

To substantiate the above findings and to determine the best source of nitrogen for the growth of the fungus, the experiment was repeated with medium I *a* in flasks. The sources of nitrogen and the amounts used were the same as in the previous trial, except that KNO_2 was also tried, while ammonium sulphate was omitted. Nitrites are usually toxic to most fungi although *Blakeslea trispora* is shown to make some growth (Leonian and Lilly, 1938). A mixture of thiamine 2 μg., riboflavine 2 μg. and biotin 0·1 μg. were added to each flask instead of malt extract.

In the controls from which nitrogen was omitted the fungus made only negligible growth, while with KNO_2 there was no growth. The dry weight of the fungus recorded on the rest of the nitrogen sources arranged in the

TABLE VI

Growth of Pellicularia koleroga *(isolate* 1) *on medium I a with different sources of nitrogen replacing* KNO_3

Nitrogen source	Growth ratings		Dry wt. of fungus in mg.
	7 days	15 days	21 days
None	*n*	*n*	1·4±0·6
Potassium nitrite ..	0	0	0·0±0·0
†Potassium nitrate ..	*	**(*)	91·6±1·4
Ammonium tartrate ..	(*)	*(*)	80·7±1·4
Asparagine	*	**	95·3±4·4
Urea	(*)	*(*)	85·7±2·6

† Readings for potassium nitrate and glucose (Table V) taken from the same set of flasks.
0: nil; *n*: negligible; * poor; ** fair; *** good; ( ): denotes less than.

decreasing order is as follows: asparagine > KNO_3 > urea >ammonium tartrate (Table VI).

Period of incubation to reach maximum growth of Pellicularia koleroga *(isolate* 1)

To medium I *a* thiamine was added at the rate of 2 μg. per flask. Since the fungus was very slow growing and its early growth being especially poor, the first harvest was taken on the 15th day and at five-day intervals thereafter.

The maximum growth was recorded on the 35th day after which autolysis was noticed (Table VII).

Optimum amount of thiamine for growth of Pellicularia koleroga *(isolate* 1)

To medium I *a* the following amounts of thiamine were added per flask: none, 0·001 μg., 0·01 μg., 0·1 μg., 1·0 μg., 2·0 μg. and 10·0 μg.

The optimum amount of thiamine for growth was found to be near 1·0 μg. per flask containing 20 ml. medium, under the cultural conditions given (Table VIII).

TABLE VII

Period of incubation in days required to reach maximum growth of Pellicularia koleroga (*isolate* 1) *with the addition of thiamine to medium I a*

Period of incubation in days	Dry wt. of fungus in mg.
15	20·4±1·5
20	36·7±2·2
25	50·0±2·5
30	74·3±4·0
35	96·6±2·3
40	84·9±2·5
Control (without thiamine at the end of 40 days)	1·8±0·4

TABLE VIII

The optimum amount of thiamine required for growth of Pellicularia koleroga (*isolate* 1) *in medium I a*

Amount of thiamine in each flask	Growth ratings		Dry wt. of fungus in mg.
	10 days	20 days	35 days
none	*n*	*n*	1·9±0·6
0·001 μg.	*n*	*n*	3·5±0·7
0·01 μg.	*n*	(*)	8·1±0·4
0·1 μg.	(*)	*(*)	44·6±2·9
1·0 μg.	*	**(*)	70·3±2·0
2·0 μg.	*	**(*)	71·8±2·0
10·0 μg.	*	**(*)	70·4±2·2

n: negligible; * poor; ** fair; *** good; ( ): denotes less than.

In an earlier experiment it was observed that when sucrose replaced glucose as carbon source in medium I *a*, both thiamine and biotin added individually to the medium promoted growth of the fungus, although better growth was obtained with thiamine (Table II). But, the amounts of the two vitamins used were not necessarily the optimum and it was possible that cultures supplied with an optimum amount of biotin would support better growth than those supplemented with an optimum amount of thiamine. This experiment was conducted to investigate this possibility and also to see whether the optimum amount of thiamine required by the fungus varied with the change in carbon source from glucose to sucrose.

To medium I *a* modified to have sucrose in place of glucose, thiamine and biotine were added individually in the following concentrations: none, 0·001 μg., 0·01 μg., 0·1 μg., 1·0 μg., 2·0 μg. and 10·0 μg.

In the *plus* thiamine series the results were identical with those obtained in the experiment with glucose as source of sugar (Tables VIII, IX) and

TABLE IX

The optimum amount of thiamine and biotin required for growth of Pellicularia koleroga (*isolate* 1) *with sucrose replacing glucose in medium I a*

Amount of growth substance in each flask	Growth ratings				Dry wt. of fungus in mg.	
	10 days		20 days		35 days	
	Thiamine	Biotin	Thiamine	Biotin	Thiamine	Biotin
none	*n*	*n*	*n*	*n*	2·2±0·2	2·2±0·2
0·001 μg.	*n*	*n*	*n*	*n*	2·5±0·2	2·7±0·1
0·01 μg.	*n*	*n*	*n*	*n*	6·9±0·1	2·8±0·1
0·1 μg.	(*)	*n*	*(*)	*n*	41·1±1·0	2·6±0·2
1·0 μg.	*	*n*	**(*)	*n*	71·1±2·1	2·7±0·4
2·0 μg.	*	*n*	**(*)	*	70·2±1·5	9·0±0·4
10·0 μg.	*	*n*	**(*)	*	69·9±0·8	10·9±0·5

n: negligible; * poor; ** fair; *** good; ( ): denotes less than.

1·0 μg. of thiamine per flask was found to be optimum. But, the almost identical values obtained on both sugars with the different amounts of thiamine was unexpected since glucose had always given better growth in earlier experiments (Tables II, V). In the *plus* biotin cultures only quantities of the vitamin above 2·0 μg. per flask gave significantly better growth, but it was in no way comparable to the growth obtained in the earlier experiment under identical conditions (Table II).

Effect of addition of thiamine, riboflavine and biotin to medium I b on growth of different isolates of Pellicularia koleroga

Isolate 1 used in these studies so far, had deteriorated steadily in culture and abruptly became useless for further experimental work. Considerable difficulty was experienced even to maintain it in a viable state on natural media. A few other isolates of the same fungus obtained from different hosts also showed different degrees of deterioration after long culturing on artificial media. None of the isolates were capable of growing on medium I *b*, unless supplemented with a small amount of malt extract (Mathew, 1954). Hence it was decided to verify whether these isolates also had similar growth substance requirements as isolate 1.

To investigate this medium I *b* was poured into test-tubes and the following growth substances were added singly: none, thiamine 2 μg., riboflavine 2 μg. and biotin 0·1 μg.

All isolates made only a negligible amount of growth in the controls and in the biotin added media, even after 30 days (Table X) incubation. Thiamine and riboflavine individually promoted good growth of isolates 3, 4, 5, 7 and 10 within 20 days while growth of isolates 2 and 12 was poor during the same period. But by the end of 30 days isolate 2 had made growth both in the presence of thiamine and riboflavine just like the other isolates. All isolates however made better and faster growth with thiamine than with riboflavine. Isolate 12 obtained from Baarn grew only in the presence of thiamine and even this growth was very slow and comparatively poor. Isolates 1, 6, 8 and 9 were in very poor state of growth and unsuitable for experimental work while isolate 11 had not been isolated at that time.

This experiment showed that many of the isolates had similar growth substance requirements as isolate 1. Isolate 7 seemed the most promising since it had shown the least changes in its rate and amount of growth from its original state, despite long subculturing in artificial media and hence this isolate was used in subsequent experiments.

TABLE X

Effect of addition of thiamine, riboflavine and biotin singly to medium I b on growth of different isolates of Pellicularia koleroga

Isolate number	Growth ratings											
	None			Thiamine 2 μg.			Riboflavine 2 μg.			Biotin 0·1 μg.		
	15 days	21 days	31 days	15 days	21 days	31 days	15 days	21 days	31 days	15 days	21 days	31 days
2	*n*	*n*	*n*	*n*	*	***(*)	*n*	*	**	*n*	(*)	(*)
3	*n*	*n*	*n*	**(*)	****	..	(*)	**(*)	..	*n*	(*)	..
4	*n*	*n*	*n*	**(*)	****	..	(*)	**(*)	..	*n*	*n*	..
5	*n*	*n*	*n*	**	***(*)	..	*n*	**	..	*n*	*n*	..
7	*n*	*n*	*n*	**(*)	****	..	(*)	**(*)	..	*n*	*n*	..
10	*n*	*n*	*n*	*(*)	***(*)	..	*n*	**	..	*n*	(*)	..
12	*n*	*n*	*n*	*n*	*	**	*n*	*n*	*n*	*n*	*n*	*n*

n: negligible; * poor; ** fair; *** good; **** excellent; ( ): denotes less than.
Only isolates 2 and 12 were observed after 21 days

Thiamine its intermediates and growth of Pellicularia koleroga (*isolate* 7)

Robbins (1938 *b*) and Schopfer (1943) have grouped fungi into different classes based on their vitamin B_1 requirements. All thiamine heterotrophic fungi do not require the thiamine molecule as such and some are able to complete the synthesis of thiamine if supplied with both its intermediates namely thiazole and pyrimidine while certain others require only any one of the two moieties (Schopfer, 1937, 1938; Schopfer and Jung, 1937; Müller and Schopfer, 1937; Sinclair, 1937; Robbins and Kavanagh, 1937, 1938 *a*, *b*, *c*; Robbins, 1938 *a*; Norkrans, 1950; Mathew, 1953).

This experiment was designed to test the effect of thiamine and its intermediates on the growth of *P. koleroga*. Thiazole and pyrimidine were added in equimolar proportions. To medium I *a* in flasks the different additions were made: none, thiazole 0·850 μg., pyrimidine 1·252 μg., thiazole *plus* pyrimidine and thiamine 2 μg.

When pyrimidine and thiazole were supplied together growth of the fungus was as good as that obtained with intact thiamine (Table XI). Pyrimidine alone, promoted a fair amount of growth while growth with thiazole alone although poor was superior to the controls.

TABLE XI

Effect of addition of thiamine and its intermediates to medium I a, on growth of Pellicularia koleroga (*isolate* 7)

Growth substance	Growth ratings		Dry wt. of fungus in mg.
	10 days	15 days	25 days
None	*n*	*n*	2·5±0·4
Thiazole 0·850 μg. ..	*n*	*n*	8·5±0·0
Pyrimidine 1·252 μg. ..	*n*	*	20·8±1·0
Thiazole+pyrimidine ..	*	**	47·7±2·8
Thiamine 2 μg. ..	*	**	48·6±0·8

n : negligible; * poor; ** fair.

I fluence of carbon source on response of Pellicularia koleroga (*isolate* 7) *to growth substances*

It was reported how isolate 1 would grow in the presence of both thiamine and biotin if the carbon source was sucrose, but only with thiamine if glucose was used (Table II). Such variations in growth could be expected with other carbon sources and with a different isolate.

Medium I *b* was taken as standard with 2% glucose and in the other treatments this was replaced by equal amounts of the other sugars. The fungus was grown on agar slants to which the following growth substances were added per tube: none, thiazole 0·850 μg., pyrimidine 1·252 μg., thiazole *plus* pyrimidine, thiamine 2 μg., riboflavine 2 μg. and biotin 1 μg. The results are summarised in Table XII.

Thiamine and the thiazole *plus* pyrimidine mixture promoted excellent growth in all tubes, the rate of growth with the latter being faster during the first week. Similar instances of greater response to the two components of thiamine in combination than with thiamine were reported in other fungi (Tatum and Bell, 1946; Norkrans, 1950). There was some response to pyrimidine and thiazole individually as well as biotin and riboflavine in all tubes, growth with pyrimidine being generally superior to the rest.

TABLE XII

Effect of addition of growth substances on growth of Pellicularia koleroga *(isolate 7) with different sources of carbon replacing glucose in medium I b*

Carbon source	Growth ratings													
	None		Thiamine 2 μg.		Thiazole 0·850 μg.		Pyrimidine 1·252 μg.		Thiazole + pyrimidine		Riboflavine 2 μg.		Biotin 1 μg.	
	7 days	21 days	7 days	21 days	7 days	21 days	7 days	21 days	7 days	21 days	7 days	21 days	7 days	21 days
Mannose ..	*n*	*n*	**	****	*n*	(*)	*n*	**	**(*)	****	*n*	*(*)	*n*	*(*)
Galactose ..	*n*	*n*	**	****	*n*	**	*n*	**	**(*)	****	*n*	**	*n*	*
Maltose ..	*n*	*n*	**	****	*n*	*	*n*	***	**(*)	****	*n*	**	*n*	*(*)
Sucrose ..	*n*	*n*	*(*)	***(*)	*n*	**	*n*	***(*)	**(*)	***(*)	*n*	*	*n*	*

n: negligible; * poor; ** fair; *** good; **** excellent; (): denotes less than.

Influence of nitrogen source on response of Pellicularia koleroga *(isolate 7) to growth substances*

In this experiment a change in the composition of the medium I *b* was brought about by using different sources of nitrogen calculated to give the same amount of nitrogen in all media. The same growth substances used in the experiment above were added in the same amounts. The results are given in Table XIII.

TABLE XIII

Effect of addition of growth substances on growth of Pellicularia koleroga *(isolate 7) with different sources of nitrogen replacing* KNO_3 *in medium I b*

Nitrogen source	Growth ratings (21 days)						
	None	Thiamine 2 μg.	Thiazole 0·850 μg.	Pyrimidine 1·252 μg.	Thiazol + pyrimidine	Riboflavine 2 μg.	Biotin 1 μg.
Potassium nitrate ..	*n*	***	*	*(*)	***	*n*	*n*
Ammonium tartrate ..	*n*	***(*)	*n*	*	***(*)	*n*	*n*
Asparagine	*n*	***(*)	(*)	*	***(*)	*n*	*n*

n: negligible; * poor; ** fair; *** good; (): denotes less than.

The fungus responded very well to thiamine and its two components in combination. Response to pyrimidine and thiazole separately was poor while growth in presence of riboflavine and biotin was negligible.

Thiamine its intermediates and growth of some isolates of Pellicularia koleroga

Whether the different isolates had similar ability as isolate 7 (Table XI) to grow in the presence of thiamine and its intermediates was tested. The treatments were: none, thiazole 0·850 μg., pyrimidine 1·252 μg., thiazole *plus* pyrimidin. and thiamine 2 μg.

All isolates grew well in tubes receiving thiamine and its two intermediates in combination. Response to thiazole was poor, but all isolates tried made some growth with pyrimidine (Table XIV).

TABLE XIV

Effect of addition of thiamine and its intermediates to medium I b on growth of different isolates of Pellicularia koleroga

Isolate number	Growth ratings (21 days)				
	None	Thiazole 0·850 μg.	Pyrimidine 1·252 μg.	Thiazole+ pyrimidine	Thiamine 2 μg.
3	*n*	*	**	****	****
4	*n*	*	**	****	****
5	*n*	*n*	(*)	**(*)	**(*)
7	*n*	*	*(*)	***	***
10	*n*	*n*	*	**(*)	**(*)

n: negligible; * poor; ** fair; *** good; **** excellent; ( ) : denotes less than.
Isolates 1, 2, 6, 8, 9 and 12 were no more viable while 11 had not been isolated.

Influence of glucose and sucrose on response of Pellicularia koleroga (*isolate* 7) *to thiamine, riboflavine and biotin*

The response of isolate 1 to thiamine and biotin varied with the carbon source (Table II). Whether isolate 7 also would behaved similarly on the two sugars was investigated. In this experiment it was also verified whether the fungus would show any increased growth with the addition of riboflavine and biotin to media containing sufficient thiamine. To basal medium I *a* in flasks, having glucose or modified to have sucrose as the source of

carbon the following additions were made: none, thiamine 2 μg., riboflavine 2 μg., biotin 1 μg., thiamine + riboflavine, thiamine + biotin, riboflavine + biotin, and thiamine + riboflavine + biotin.

On both media thiamine alone was as effective as any other combinations of the three vitamins tried, while riboflavine and biotin individually and in combination promoted only a poor growth, although slightly superior to the controls (Table XV). On media containing thiamine, glucose supported better growth than sucrose.

TABLE XV

Effect of addition of thiamine, riboflavine and biotin to medium I a with glucose and sucrose as carbon sources, on growth of Pellicularia koleroga *(isolate 7)*

Growth substance	Growth ratings				Dry wt. of fungus in mg.	
	10 days		15 days		25 days	
	Glucose	Sucrose	Glucose	Sucrose	Glucose	Sucrose
None	*n*	*n*	*n*	*n*	2·4±0·4	2·0±0·5
Thiamine 2 μg.	*	*	**	*(*)	53·4±2·0	44·4±2·8
Riboflavine 2 μg.	*n*	*n*	*n*	*n*	7·4±0·5	6·5±0·9
Biotin 1 μg.	*n*	*n*	*n*	*n*	5·5±0·8	6·7±0·4
Thiamine +riboflavine ..	*	*	**	*(*)	56·8±0·4	43·2±3·2
Thiamine +biotin	*	*	**	*(*)	53·8±3·5	43·5±1·7
Riboflavine+biotin ..	*n*	*n*	*n*	*n*	7·0±1·5	7·9±0·9
Thiamine+riboflavine+biotin	*	*	**	*(*)	54·4±2·4	43·4±1·4

n: negligible; * poor; ** fair; ( ): denotes less than.

Effect of addition of yeast extract, casein hydrolysate and growth substances to medium I a on growth of Pellicularia koleroga *(isolate 7)*

Many vitamin heterotrophic fungi are known to grow better with the addition of some natural product like malt or yeast extract to the medium than when a mixture of pure growth substances are added. This may be

due to the buffering action of the extracts on the medium, or due to the presence of essential trace elements, amino acids and unknown growth substances in the extract (Schopfer, 1943; Fries, 1948). In naturally occurring filamentous fungi total amino acid deficiency is very rare, although some fungi are reported to make very little growth in their absence or show marked increase in growth in the presence of small amounts (Mosher *et al.*, 1936; Robbins, Mackinnon and Ma, 1942; Robbins and Ma, 1945; Cantino, 1948; Norkrans, 1950).

TABLE XVI

Effect of addition of yeast extract, casein hydrolysate and growth substances to medium I a, on growth of Pellicularia koleroga (*isolate* 7)

Substance added	Growth ratings		Dry wt. of fungus in mg.
	10 days	15 days	21 days
None	*n*	*n*	1·4±0·3
Thiamine	(*)	*(*)	22·7±1·0
Thiamine+vitamin mixture ..	(*)	*(*)	23·6±2·4
Casein hydrolysate	*n*	*n*	1·6±0·4
Thiamine+casein hydrolysate ..	(*)	**	47·7±5·3
Thiamine+vitamin mixture+ casein hydrolysate	(*)	**	41·2±3·1
Yeast extract	*(*)	**	26·1±1·7

n: negligible; * poor; ** fair; ( ): denotes less than.

Vitamin mixture riboflavine, pyridoxine, pantothenic acid, nicotinic acid, biotin, *p*-aminobenzoic acid each 1 μg. and inositol 1 mg.

Whether yeast extract and casein hydrolysate would increase the growth of the fungus significantly over that obtained with thiamine alone or thiamine *plus* a vitamin mixture (riboflavine, pyridoxine, pantothenic acid, nicotinic acid, biotin, *p*-aminobenzoic acid each 1 μg. and inositol 1 mg.) was investigated. Acid hydrolysed casein was prepared from vitamin free casein and dilute yeast extract solution from yeast powder. To medium I *a* in flasks, the following treatments were given: none, casein hydrolysate

6 mg., thiamine 2 μg., thiamine + vitamin mixture, thiamine + casein hydrolysate, thiamine + vitamin mixture + casein hydrolysate and yeast extract 2 ml.

Thiamine alone was as good as the thiamine *plus* vitamin mixture but in the presence of thiamine casein hydrolysate considerably increased the growth and at the end of 21 days it was twice as that obtained with thiamine alone. Yeast extract supported the best growth during the first 10 days autolysis was noticed before the 15th day and when dry weight was determined on the 21st day it was not much more than that obtained with thiamine. This was obviously due to the essential metabolites in the dilute yeast extract solution being insufficient and exhausted at an early stage (Table XVI).

Inhibitory effects of vitamins

Vitamins have been shown to have inhibitory effect—especially in excessive doses—on growth of certain fungi (Schopfer, 1935; Barnett and Lilly, 1948; Elliott, 1949; Mathur, Lilly and Barnett, 1950). The tolerance of this fungus to the three principal vitamins, thiamine, riboflavine and biotin used in these studies was investigated.

Influence of high concentrations of thiamine, riboflavine and biotin on growth of Pellicularia koleroga *(isolate* 7)

Biotin is the most active of all the known growth substances and is still active even at a dilution of 1 : 400,000,000,000 (Hawker, 1950). Lilly and Barnett (1951) found the optimum amount of biotin to be 1/20th of thiamine and pyridoxine and 1/1,000th that of inositol for a number of fungi. However, experiments conducted so far with this fungus showed that biotin contrary to expectations was active only in comparatively large doses (Tables II, IX, X). This needed further verification and since nothing was known about the optimum level of riboflavine for growth of the fungus, this was also investigated. Riboflavine was added to the medium I *a* in flasks and biotin to the same medium modified to have sucrose in place of glucose.

Comparatively large amounts of 5 and 10 μg. per 20 ml. of medium, of both vitamins were found necessary to slightly improve the growth over the controls (Table XVII). Riboflavine, however, inhibited some growth at the higher level.

The effects of high concentrations of thiamine and biotin on the growth of the fungus was studied in another experiment. To medium I *b* in tubes

TABLE XVII

Effect of addition of different amounts of riboflavine to medium I a and biotin to medium I a modified to have sucrose in place of glucose on growth of Pellicularia koleroga (*isolate* 7)

Amount of growth substance in each flask	Dry wt. of fungus in mg. (25 days)	
	Glucose+riboflavine	Sucrose+biotin
none	1·2±0·4	1·7±0·3
1·0 μg.	1·7±0·1	1·8±0·1
5·0 μg.	10·1±1·2	5·5±1·0
10·0 μg.	4·9±0·8	9·5±1·8

the following amounts of thiamine were added: none, 1·0 μg., 10·0 μg., 50·0 μg. and 100·0 μg. A depression in growth was noticed only at the very high concentration of 100·0 μg. per tube which works out to 20,000 μg. per litre.

To study the effect of biotin, medium I *b* modified to have maltose in place of glucose was used since in an earlier experiment the best response to biotin was obtained with maltose as carbon source (Table XII). The treatments per tube were: none, 1·0 μg., 10·0 μg. and 20·0 μg. The growth was negligible in the controls and at 1·0 μg. level. There was good response at the higher levels and growth was not inhibited even at 20·0 μg. per tube (4,000 μg. per litre).

Influence of a sub-optimal amount of thiamine on response of Pellicularia koleroga (*isolate* 7) *to other growth substances*

Since the fungus had on various occasions responded to a certain extent to growth substances other than thiamine also, it was considered interesting to investigate whether they would promote still better growth of the fungus if a sub-optimum amount of thiamine was supplied as a 'starter.' Medium I *b* in tubes were divided into two lots and one set received 0·01 μg. of thiamine per tube, while the others were kept as controls. To both series the following additions of growth substances were made individually: none, riboflavine 2 μg., pyridoxine 2 μg., pantothenic acid 2 μg., nicotinic acid 2 μg., biotin 2 μg., inositol 2 mg. and *p*-aminobenzoic acid 2 μg.

No reliable conclusions could be drawn since in all tubes receiving the sub-optimal amount of 0·01 μg. of thiamine there was a fair growth and it was difficult to judge visually whether there was improved growth due to the other growth substances; also, growth in some of the replicates of the same treatment was not equal. For greater accuracy a quantitative experiment in liquid culture will prove necessary.

However, the experiment yielded another interesting and unexpected result; pantothenic acid singly promoted a fair amount of growth in the control series without thiamine.

Influence of carbon source on response of Pellicularia koleroga (*isolate* 11) *to growth substances*

The response of the fungus to thiamine and biotin was shown to vary with the carbon source (Table II). On addition of biotin to the medium the fungus grew if sucrose was the carbon source but not with glucose. Dr. S. D. Garrett in a communication suggested that the fungus might respond to biotin with fructose also as carbon source, since sucrose on hydrolysis is converted into glucose and fructose. But, it was also observed that as the cultures were maintained for a long time in the laboratory, their response to biotin was reduced considerably (Tables II, IX). Again, the experiment described above showed that isolate 7 grew on addition of pantothenic acid to the medium while isolate 1 did not respond to it (Table II) and this was the first suggestion that all isolates of *P. koleroga* were not necessarily physiologically alike in all respects. Till then, it was assumed that all isolates were physiologically identical, based on results of experiments described in an earlier paper (Mathew, 1954) as well as results described in this (Table X). The slight differences observed between isolates on some occasions were thought to be due to changes they have undergone while in culture. To clear all these doubts it was deemed best to use a fresh isolate of the fungus.

To medium I *b* and the same modified to have fructose and sucrose also as carbon sources the following treatments were applied: none, thiazole 0·850 μg., pyrimidine 1·525 μg., thiazole *plus* pyrimidine, thiamine 2 μg., riboflavine 2 μg., pyridoxine 2 μg., pantothenic acid 2 μg., nicotinic acid 2 μg., biotin 2 μg., inositol 2 mg. and *p*-aminobenzoic acid 2 μg. The tubes were inoculated with a fresh isolate, isolated a month before.

The growth of the fungus was negligible in all treatments except those receiving thiazole, thiamine and the combination of thiazole *plus* pyrimidine. Growth was excellent with thiamine and its intermediates in combination on all three sugars. Thiazole promoted a fair amount of growth with glucose

and a poor growth with fructose as carbon sources, but growth was negligible with sucrose.

DISCUSSION

The fungus *Pellicularia koleroga* Cooke was morphologically identical on all its numerous hosts found in Mysore; cultural studies revealed that the different isolates of the fungus were also morphologically and physiologically alike (Mathew, 1954). When the investigations reported in this paper were begun, it was assumed from the earlier observations that what is true for any one isolate will be generally true for the entire species. During the course of this work further evidence was obtained to support this view. None of the isolates tried was capable of growing in pure synthetic media, but all of them readily responded to thiamine and its two moieties in combination (Table XIII). Many of them also responded to pyrimidine and thiazole separately as well as to riboflavine and biotin (Tables II, X, XI, XII, XIII, XIV). A certain amount of difference was noticed between isolates in their response to these growth substances (Tables X, XIV), but there was also evidence to believe that this might be due to changes the isolates have undergone while in culture, resulting in loss of some of their original capacities for synthesis (Tables II, IX). Only at a very late stage of the studies, evidence was obtained to suggest that all isolates were not necessarily physiologically alike in all respects, although there was general agreement. Most of the isolates tried had greater ability to synthesise thiamine from pyrimidine than with thiazole (Table XIV) and some ability to grow in presence of riboflavine (Table X), but at least one isolate was observed to differ. Similarly isolate 7 was shown to respond to pantothenic acid while isolate 1 did not (Table II). Although, growth substance deficiencies are genetically determined characters generally true for the entire species (Schopfer, 1943), as is well illustrated by 215 single cell isolates of *Ustilago violacea* from 9 morphologically different varieties all having identical growth substance requirements (Blumer, 1940), instances of different isolates or strains of the same fungus having different growth substance requirements are also known (Hawker, 1939 *a*, Robbins and Ma, 1941; Andes, 1946; Lilly and Barnett, 1948).

One of the characteristics of a true growth substance for a fungus is its marked specificity (Schopfer, 1943). This holds good for most fungi and the reader is referred to Robbins and Kavanagh (1942) for the numerous examples. However, many micro-organisms unlike animals have greater synthetic ability and hence are less specific and respond to any one of the moieties of a vitamin as in the case of thiamine or even to compounds closely related to the vitamin (Schopfer, 1943; Lilly and Barnett, 1951).

Similar response to the moieties of thiamine have been shown in isolates of *P. koleroga* also (Tables XI, XIV). More difficult to understand are the rare instances of one vitamin apparently replacing another. Schopfer (1943) termed this as the ' replacement of factors ' and according to him *Staphylococcus aureus* requires 0·05 μg. each of biotin, thiamine and nicotinic acid for maximum growth, but the same amount of growth can also be obtained by omitting biotin and increasing the amounts of thiamine and nicotinic acid each to 5 μg., higher amounts of the latter two vitamins replacing biotin. An instance involving excessive doses is that of *Phycomyces*, where growth equal to that obtained with 0·5 μg. each of pyrimidine and thiazole is possible with 10 μg. of pyrimidine and 1,000 μg. of β-alanine also (Robbins, 1940). Still remarkable is the case of *Trichophyton interdigitale* (Mosher *et al.*, 1936) which requires a combination of thiamine, riboflavine, inositol and pantothenic acid for maximum growth, but is capable of growing if any one of them is supplied; it does not grow in the controls. These authors also observed that the early comparative ratings (8 days) were more significant and that after longer periods of incubation growth was generally equal in most treatments. This led to the conclusion that the fungus synthesises growth substances as it grows and that if any one of the vitamins is supplied it has the ability to synthesise the rest of its needs; some evidence to support this was obtained. Robbins and Ma (1942) with the use of suitable heterotrophic fungi have demonstrated that a number of fungi investigated synthesised other vitamins when supplied with deficient vitamin.

Some isolates of *P. koleroga* were not very much different from *T. interdigitale* in that thiamine, riboflavine, biotin and pantothenic acid each separately promoted their growth (Tables II, X, XII) and that the early growth ratings were often of great significance. However, they differed in other respects. Although, *T. interdigitale* would grow with each of the four vitamins separately it required all of them in combination to reach maximum growth. On the contrary, the maximum possible growth of *P. koleroga* was obtained with thiamine alone and additions of other growth substances to media containing enough thiamine had no effect (Table XV). All isolates of the fungus have readily responded to thiamine throughout, under different cultural conditions. But, response to riboflavine, biotin and pantothenic acid varied among the different isolates as well as in the same isolate with changes in the composition of the medium (Tables II, X, XII, XIII) and on no occasion was comparable to that of thiamine. It was also noticed that ability of the fungus to grow with vitamins other than thiamine varied in different experiments under identical conditions; the longer they were in culture the lesser the ability (Tables II, IX).

These results may be explained as follows: All isolates of the fungus are deficient for thiamine and if it is given they synthesise all other requirements. But, ability to synthesise thiamine is not completely lost and the fungus synthesises thiamine from its two moieties in combination and grows as well as with thiamine. Many isolates also have ability to slowly synthesise thiamine from any one or both of the components separately, but this capacity varies considerably with the composition of the medium especially the carbon source. In addition, some isolates synthesise thiamine slowly if either riboflavine, biotin or pantothenic acid is supplied and this capacity also varies with a change in the carbon source. The ability to synthesise thiamine in the presence of other growth substances is considerably reduced as the cultures are maintained for long periods in laboratory media.

The catalytic and quantitative actions which are also characteristics of true growth substances (Schopfer, 1943) could be demonstrated only with respect to thiamine (Tables VIII, IX, XV, XVII). In some experiments 500 to 4,000 μg. of biotin per litre was found necessary even to promote a limited growth (Tables IX, XVII) and it would be difficult to reconcile if biotin which is active in greater dilution than any other vitamin was to be considered a true growth substance for this fungus. As explained earlier, it is more likely that slow synthesis of thiamine is accomplished somehow in the presence of comparatively large amounts of biotin; this applies to other growth substances also.

The discussion logically leads to the loss of original abilities for synthesis exhibited by some of the isolates during the course of the investigations and to its causes. A close scrutiny of all results obtained with isolates 1 and 7 show how these isolates in the beginning had ability to synthesise thiamine slowly under a number of cultural conditions but later required either intact thiamine or both its intermediates in combination (Tables II, IV, IX, XII, XIII, XV).

A somewhat parallel instance where wide fluctuations were observed between experiments conducted at different times with the same isolates, was reported by Lilly and Barnett (1948) in the case of 14 single spore isolates of *Lenzites trabea* obtained from the same fruit body. These authors were able to observe coloured sectors and pigmentation in the different isolates, after maintaining them in malt agar for some time and whenever coloured sectors arose abrupt changes were noticed. Others have also shown that when physiological changes were noticed in the same isolate during the course of a series of experiments it was due to saltations or mutations (Brown, 1926; Mohendra, 1928; Robbins and Ma, 1941; Hansen and

Snyder, 1943; Hawker, 1947 *b*). This phenomenon is indeed familiar even to the casual student of Fusaria. Certain rich media like Richard's solution, high temperature and poisons are known to favour mutations in fungi, while in some cases anastomosis leads to genuinely new forms (Brown, 1926; Hawker, 1950; Gaümann, 1950). Anastomosis is very frequently observed both in nature and in cultures of *P. koleroga* and this may explain the occurrence of isolates having slightly altered growth requirements. It is also possible that inhibitory substances in the sample of malt extract used (Mathew, 1954) might have favoured mutation. Vitamin deficiencies have also been induced by X-rays and Ultraviolet radiations in *Neurospora* (Beadle and Tatum, 1945) and *Ophiostoma* (Fries, 1945) and many of these were shown to differ by a single gene from the wild type. There is every reason to suppose that similar mutations resulting in altered growth substance requirements occur both in nature and in culture.

An opposite case of what he described as progressive changes leading towards heterotrophism was reported by Schopfer (1943). *Ustilago violacea* has the ability to condense thiazole and pyrimidine in combination, to thiamine. But in the course of experiments strains which no longer had this ability in full showed a reduction in growth obtained with pyrimidine *plus* thiazole compared to thiamine. Even more interesting is the converse case of *Rhodotorula rubra* reported by Schopfer (1943), where the fungus when grown for 14 months on a thiamine free medium gradually improved growth by 3 to 4 times, although a poor grower to start with. But Hansen and Snyder (1943) and Hawker (1947 *b*) have shown that the loss of sporulating ability observed in *Penicillium notatum* and *Melanospora destruens* respectively, seemingly, accumulated gradually over a number of years due to saltations.

In cultures of *P. koleroga* slow cumulative as well as abrupt changes have been observed. A progressive reduction both in the rate and amount of growth was noticed in all isolates when they were in culture for a long time. In many fungi mutants or saltants can be readily recognised by change of colour, loss of sporulating ability and other such easily noticeable characters. But *P. koleroga* neither sporulates in culture nor produces any type of growth which can be readily noticed as a departure from normal. However, abrupt changes resulting in considerably reduced rate of growth were noticed in some isolates and a few of them never recovered. Others, on subculturing remained without making proper growth for some time, but suddenly seemed to reverse and this was followed by acclerated growth for one or more generations. It seemed as if the fungus was very slowly synthesising some essential metabolites.

Very often when 3 or 4 subcultures were made from the same tube only one or two grew satisfactorily. The poor growers were rejected and this sort of unintentional selection has happened in all isolates without any proper records being kept. Hence, it is doubtful whether a further continuation of studies with a few isolates still available (isolates 3, 4, 7 and 11) would answer the questions raised in the discussion. It will be necessary to carry out a meticulously planned investigation, under carefully controlled conditions with a number of fresh isolates.

The loss of capacity for synthesis of its own requirements of growth substances is not complete in the case of many isolates of *P. koleroga.* Hence, it is possible that if a large number of isolates are grown under equally large number of cultural conditions some may grow in strictly synthetic media. Instances of fungi requiring an external supply of growth substances only under certain conditions are known (Robbins and Kavanagh, 1938 *b*; Fromageot and Tschang, 1938; Stokes, Foster and Woodward, 1943; Lilly and Barnett, 1947; Barnett and Lilly, 1948). It is also possible that isolates showing response to growth substances hitherto found not favourable, may be found. Possibility of coming across isolates having reduced capacities for synthesis and requiring the intact thiamine molecule is also not remote.

SUMMARY

Pellicularia koleroga Cooke grew best at temperatures varying from 21 to 23° C. and made some growth at 7 to 8° C., but did not grow above 31 to 32° C.

Good growth of the fungus was obtained over a wide range of pH varying from 3·8 to 7·3.

The fungus readily assimilated and grew well on sources of carbon like glucose, mannose, galactose, fructose, maltose, sucrose and starch, fairly well on glycerol and mannitol, poorly on xylose and lactose and made no growth on dulcitol.

Nitrate, ammonium and organic nitrogen were used by the fungus but no growth was possible with nitrite nitrogen.

The fungus did not grow on strictly synthetic media, but grew well on addition of extracts of natural products and fungal mats, staled fungal medium and pure growth substances to the media.

All isolates tried responded to thiamine and its two intermediates namely thiazole and pyrimidine in combination. But, the same isolate on different media and different isolates on the same medium responded differently to thiazole and pyrimidine used separately.

Similarly, different isolates had varying capacities to grow with the addition of riboflavine, biotin and pantothenic acid to the medium. For the same isolate these abilities varied with the composition of the medium. It is believed that in the presence of the above growth substances the fungus is able to slowly synthesise thiamine.

On repeated sub-culturing in artificial media all isolates became more and more dependant on thiamine, while response to the other growth substances was reduced. However, a progressive reduction in the rate and amount of growth was noticed even in the presence of thiamine.

The optimum amount of thiamine for the growth of the fungus determined at the period when the fungus reaches maximum growth, was found to be near 1·0 μg. per 20 ml. of medium. Comparatively large amounts of biotin and riboflavine were found necessary to promote some growth of the fungus, especially after long maintenance in artificial media.

Additions of other growth substances to medium containing optimum amount of thiamine had no effect, but casein hydrolysate increased growth considerably.

The growth of the fungus was not inhibited by excessive doses of thiamine and biotin, while riboflavine in similar doses depressed growth.

The occurrence of different isolates having different capacities for synthesis and also the loss of some of the original capacities for synthesis exhibited by some isolates in culture are very likely due to mutations.

Acknowledgements

I am deeply indebted to Professor T. S. Sadasivan, Director, University Botany Laboratory, Madras, for suggesting the problem and the keen interest he took throughout the work and in the preparation of this article. I wish to thank Dr. C. V. Subramanian, Senior Lecturer, University Botany Laboratory, for advise both during the investigations and in writing this paper. I am grateful to the University of Madras, for the award of a studentship.

References

Andes, J. O. .. "Effect of growth substances on *Glomerella cingulata*," *Phytopathology*, 1946, **36**, 394.

Asthana, R. P. and Hawker, L. E. "The influence of certain fungi on the sporulation of *Melanospora destruens* Shear and of some other Ascomycetes," *Ann. Bot.*, 1936, **50**, 325–44.

Barnett, H. L. and Lilly, V. G... "The interrelated effects of vitamins, temperature and pH on vegetative growth of *Sclerotinia camelliæ*," *Amer. J. Bot.*, 1948, **35**, 297–302.

Beadle, G. W. and Tatum, E. L. "*Neurospora*, II. Methods of producing and detecting mutations concerned with nutritional requirements," *Ibid.*, 1945, **32**, 678–86.

*Blumer, S. .. "Über die Aneurinheterotrophie bei einigen antheren bewohnenden Brandpilzen," *Mitt. Naturforsch. Ges. Bern.*, 1940, p. 19.

Brannon, J. M. .. "Influence of glucose and fructose on growth of fungi," *Bot. Gaz.*, 1923, **76**, 257–73.

Brown, W. .. "Studies in the genus *Fusarium*, IV. On the occurrence of saltations," *Ann. Bot.*, 1926, **40**, 223–43.

Cantino, E. C. .. "The vitamin nutrition of an isolate of *Blastocladia pringsheimii*," *Amer. J. Bot.*, 1948, **35**, 238–42.

Corum, C. J. .. "Carbohydrate nutrition of *Rhizopus suinus*," *Bot. Gaz.*, 1942, **103**, 740–50.

*Elliott, E. S. .. "Effect of vitamins on growth of some graminicolus species of *Helminthosporium* and *Fusarium*," *Proc. West. Va. Acad. Sci.*, 1949, **20**, 65–68.

Farries, E. H. M. and Bell, A. F. "On the metabolism of *Nematospora gossypii* and related fungi with special reference to the source of nitrogen," *Ann. Bot.*, 1930, **44**, 423–55.

Fries, N. .. "X-ray induced mutations in the physiology of *Ophiostoma*," *Nature*, 1945, **156**, 757.

———— .. "The nutrition of fungi from the aspect of growth factor requirements," *Trans. Brit. mycol. Soc.*, 1948, **30**, 118–34.

*Fromageot, C. and Tschang, J. L. "Sur la synthèse des pigments Carotenoides par *Rhodotorula sanniei*," *Arch. Mikrobiol.*, 1938, **9**, 434–48.

Gäumann, E. .. *Principles of Plant Infection*, 1950, Crosby Lockwood and Son Ltd., London.

Hansen, H. N. and Snyder, W. C. "Relation of dual phenomenon in *Penicillium notatum* to penicillin production," *Science*, 1944, **99**, 264–65.

Hawker, L. E. .. "The effect of certain accessory growth substances on the sporulation of *Melanospora destruens* and of some other fungi," *Ann. Bot.*, 1936, **50**, 699–718.

———— .. "The nature of the accessory growth factors influencing growth and fruiting of *Melanospora destruens* Shear and of some other fungi," *Ibid.*, N.S., 1939 *a*, **3**, 657–76.

———— .. "The influence of various sources of carbon on the formation of perithecia by *Melanospora destruens* Shear in the presence of accessory growth factors," *Ibid.*, N.S., 1939 *b*, **3**, 455–68.

———— .. "Further experiments on growth and fruiting of *Melanospora destruens* Shear in the presence of various carbohydrates, with special reference to the effects of glucose and sucrose," *Ibid.*, N.S., 1947 *a*, **11**, 245–59.

———— .. "Effects of temperature of storage on the rate of loss of fertility of stock cultures of *Melanospora destruens*," *Nature*, 1947 *b*, **159**, 136.

Hawker, L. E. .. "The effect of certain growth substances on mycelial growth and fruiting of *Melanospora destruens* Shear," *Trans. Brit. mycol. Soc.*, 1948, **30**, 135–40.

———— .. *Physiology of Fungi*, 1950, University of London Press Ltd., London.

——— and Chaudhri, S. D. .. "Growth and fruiting of certain Ascomycetous fungi as influenced by the nature and concentration of the carbohydrate in the medium," *Ann. Bot.*, N.S., 1946, **10**, 185-94.

Hawkins, L. A. .. "The utilisation of certain pentoses and compounds of pentoses by *Glomerella cingulata*," *Amer. J. Bot.*, 1915, **2**, 375–89.

Horr, W. H. .. "Utilisation of galactose by *Aspergillus niger* and *Penicillium glaucum*," *Plant Physiol.*, 1936, **11**, 81–99.

*Kögl, F. and Fries, N. .. "Über den Einfluss von Biotin, Aneurin und Meso-Inosit auf das Wachstum verschiedener Pilzarten," *Z. Physiol. Chem.*, 1937, **249**, 93–110.

Leben, C. and Keitt, G. W. .. "*Venturia inæqualis* (Cke.) Wint., V. The influence of carbon and nitrogen sources and vitamins on growth *in vitro*," *Amer. J. Bot.*, 1948, **35**, 337-43.

Leonian, L. H. and Lilly, V. G. "Studies on the nutrition of fungi, I. Thiamine its constituents and the source of nitrogen," *Phytopathology*, 1938, **28**, 531–48.

———— .. "Auxithals synthesised by some filamentous fungi," *Plant Physiol.*, 1940, **15**, 515–25.

Lilly, V. G. and Barnett, H. L. "The influence of pH and certain growth factors on mycelial growth and perithecial formation of *Sordaria fimicola*," *Amer. J. Bot.*, 1947, **34**, 131–38.

———— .. "The inheritance of partial thiamine deficiency in *Lenzites trabes*," *J. agric. Res.*, 1948, **77**, 287–300.

———— .. *Physiology of the Fungi*, 1951, McGraw-Hill Book Company, New York.

*Mathur, R. S., Barnett, H. L. and Lilly, V. G. "Sporulation of *Colletotrichum lindemuthianum* in culture," *Phytopathology*, 1950, **40**, 104–14.

Mathew, K. T. .. "Growth factor requirements of *Pellicularia koleroga* Cooke in pure culture," *Nature*, 1952, **170**, 889–90.

———— .. "Thiamine its intermediates and growth of *Corticium microsclerotia* (Matz.) Weber," *Proc. Ind. Acad. Sci.*, Sec. B., 1953, **38**, 1–9.

———— .. "Studies on the Black Rot of Coffee, I. The disease in South India and some general considerations," *Ibid.*, 1954, **39**, 133–70.

Matsumoto, T. and Yamamoto, W. "*Hypochnus sasakii* Shirai in comparison with *Corticium stevensii* Burt and *Corticium koleroga* (Cooke) v. Hohn.," *Trans. Nat. Hist. Soc. Formos.*, 1935, **25**, 161–75.

Mitchell, H. K. and Houlahan, M. B. "*Neurospora*, IV. A Temperature sensitive riboflavineless *mutant*," *Amer. J. Bot.*, 1946, **33**, 31–35.

Mohendra, K. R. .. "A study of the changes undergone by certain fungi in artificial culture," *Ann. Bot.*, 1928, **42**, 863–89.

Mosher, W. A., Saunders, D. H., Kingsley, L. B. and Williams, R. J. "Nutritional requirements of the pathogenic mold *Trichophyton interdigitale*," *Plant Physiol.*, 1936, **11**, 795–806.

*Müller, W. F. and Schopfer, W. H. "L'action de l'aneurine et de ses constituents sur *Mucor ramannianus* Möll.," *C.R. Acad. Sci.*, Paris, 1937, **205**, 687–89.

Narasimhan, M. J. .. "Black Rot of Coffee in Mysore," *Phytopathology*, 1933, **23**, 875–86.

Norkrans, B. .. "Studies in growth and cellulolytic enzymes of *Tricholoma* with special reference to mycorrhiza formation," *Symb. Bot. Ups.*, 1950, **11**, 1–126.

Ramakrishnan, T. S. .. "Studies in the genus *Colletotrichum*, II. Physiological studies on *Colletotrichum falcatum* Went.," *Proc. Ind. Acad. Sci.*, Sec. B, 1941, **14**, 395–411.

Robbins, W. J. .. "The assimilation by plants of various forms of nitrogen," *Amer. J. Bot.*, 1937, **24**, 243–50.

———— .. "Thiamine and growth of species of *Phytophthora*," *Bull. Torrey bot. Club*, 1938 *a*, **65**, 267–70.

———— .. "Organisms requiring vitamin B_1," *Proc. Nat. Acad. Sci.*, U.S.A., 1938 *b*, **24**, 53–56.

———— .. "Response of excised tomato roots to B (-4-methylthiazole-5-)-alanine," *Plant Physiol.*, 1940, **15**, 547–52.

——— and Kavanagh, F. .. "Intermediates of vitamin B_1 and growth of *Phycomyces*," *Proc. Nat. Acad. Sci.*, U.S.A., 1937, **23**, 499–502.

———— .. "Intermediates of vitamin B_1 and the growth of *Torula*," *Plant Physiol.*, 1938 *a*, **13**, 611–19.

———— .. "Thiamine and growth of *Phythium butleri* Bull.," *Bull. Torrey bot. Club*, 1938 *b*, **65**, 453–61.

———— .. "Vitamin B_1 or its intermediates and growth of some fungi," *Amer. J. Bot.*, 1938 *c*, **25**, 229–36.

——— and Kavanagh, V. .. "Vitamin deficiencies of the filamentous fungi," *Bot. Rev.*, 1942, **8**, 411–71.

——— and Ma, R. .. "Biotin and growth of *Fusarium avenaceum*," *Bull. Torrey bot. Club*, 1941, **68**, 446–62.

———— .. "Vitamin deficiencies of *Ceratostomella* and related fungi," *Amer. J. Bot.*, 1942, **29**, 835–43.

———— .. "Growth factors for *Trichophyton mentagrophytes*," *Ibid.*, 1945, **32**, 509–21.

——— MacKinnon, J. E. and Ma, R. "Vitamin deficiencies of *Trichophyton discoides*," *Bull. Torrey bot. Club*, 1942, **69**, 509–51.

Saksena, R. K. .. "Thiamine and growth of some species of *Phytium*," *Proc. Ind. Acad. Sci.*, Sec. B, 1941, **14**, 141–48.

Schade, A. L. .. "The nutrition of *Leptomitus*," *Amer. J. Bot.*, 1940, **27**, 376–84.

*Schopfer, W. H. .. "Les vitamines crystallisées B comme hormones de croissance chez un micro-organisme," *Arch. Mikrobiol.*, 1934, **5**, 502–10.

*Schopfer, W. H. .. "Etude sur les facteurs de croissance. Action de la cristalisee B_1 sur *Rhizopus* et d'autres Mucorinees," *Zeitschr. f. Vitaminforschung*, 1935, **4**, 187–206.

*——— .. "L'action des constituants de l'aneurine sur les levures *Rhodotorula rubra* et R. *flava*)," *C.R. Acad. Sci.*, Paris, 1937, **205**, 445.

*——— .. "La pyrimidine facteur de croissance de micro-organisme (*Rhodotorula*, Mucorinees, *Dematium*)," *Protoplasma*, 1938, **31**, 105.

——— .. *Plants and Vitamins*, 1943, Chronica Botanica Company, Waltham, Mass.

*——— and Jung, A. "L'action des produits de desintegration de l'aneurine sur *Phycomyces*," Le second facteur de croissance des Mucorinees," *C.R. Acad. Sci.*, Paris, 1937, **204**, 1500.

Sinclair, H. M. .. "Growth factors for *Phycomyces*," *Nature*, 1937, **140**, 361.

Steinberg, R. A. .. "Role of molybdenum in the utilisation of ammonium and nitrate nitrogen by *Aspergillus niger*," *J. agric. Res.*, 1937, **55**, 891–901.

——— .. "Relation of carbon nutrition to trace elements and accessory requirements of *Aspergillus niger*," *Ibid.*, 1939, **59**, 749–63.

*Stokes, J. L., Foster, J. W. and Woodward, H. B. "Synthesis of pyridoxine by a 'pyridoxine-less' X-ray mutant of *Neurospora sitophila*," *Arch. Biochem.*, 1943, **2**, 235–45.

Studt, B. .. "Morphological and physiological studies of a strain of *Candida albicans* associated with vaginitis," *Amer. J. Bot.*, 1941, **28**, 509–15.

Tatum, E. L. and Bell, T. T. .. "*Neurospora*. III. Biosynthesis of thiamine", *Ibid.*, 33, 15–20.

*Articles not seen in original.

STUDIES IN THE CHENOPODIACEÆ

II. Embryology of *Arthrocnemum indicum* Moq.

By T. S. Mahabale,* F.A.Sc.
(Institute of Science, Bombay)
AND
I. N. Solanky
(M. T. B. College, Surat)

Received March 21, 1953

Introduction

Arthrocnemum indicum Moq. is a perennial herb with prostrate stem giving out numerous erect or ascending, fleshy, short jointed, leafless branches. The flowers are minute, hermaphrodite, and form cone-like spikes. There is only one stamen with large oblong anther. The seed is trigonous and compressed with thin yellow testa. The plant is a mangrove like *Suæda maritima.* The flowers were collected from Dandi, a village near Surat.

References to previous literature on the family have been given in Part I. These are mainly on *Chenopodium Bonus Henricus* (Souèges, 1920), *Beta vulgaris* (Artschwager, 1927; Artschwager and Starret, 1933; and Oksijuk, 1927), *Kochia scoparia* (Williams, 1932), *Atriplex hynænelytra* (Billings, 1934), *Chenopodium album* (Bhargava, 1936), *Suæda fruticosa* (Mahabale and Solanky, 1953), and on a few other plants of Chenopodiaceæ (Cooper, 1935). A reference to Schnarf (1927) and other literature on the Chenopodiaceæ showed that no work has been done on *Arthrocnemum indicum.* It was, therefore, thought worthwhile to work out its embryology.

The material was fixed in formalin-acetic-alcohol and Nawaschin's fluid, of which the latter gave good results. The customary methods of dehydration and embedding in paraffin were followed. Sections were cut at 10–18 μ thick and stained with iron-hæmatoxylin which proved satisfactory.

Description

Floral organogeny.—The development of floral organs is strictly acropetal as in *Beta vulgaris* (Artschwager, 1927), *Chenopodium album* (Bhargava, 1936) and *Suæda fruticosa* (Mahabale and Solanky, 1953). An individual flower primordium appears as a conical projection (Fig. 1) on which perianth

* Present address : Botany Department, University of Poona, Poona-7.

soon appears (Fig. 2). Stamen primordium appears next (Fig. 3) followed by carpels (Fig. 4). The carpels grow upwards (Fig. 5) and enclose the ovule (Fig. 6). Usually there is only one stamen but in some cases two.

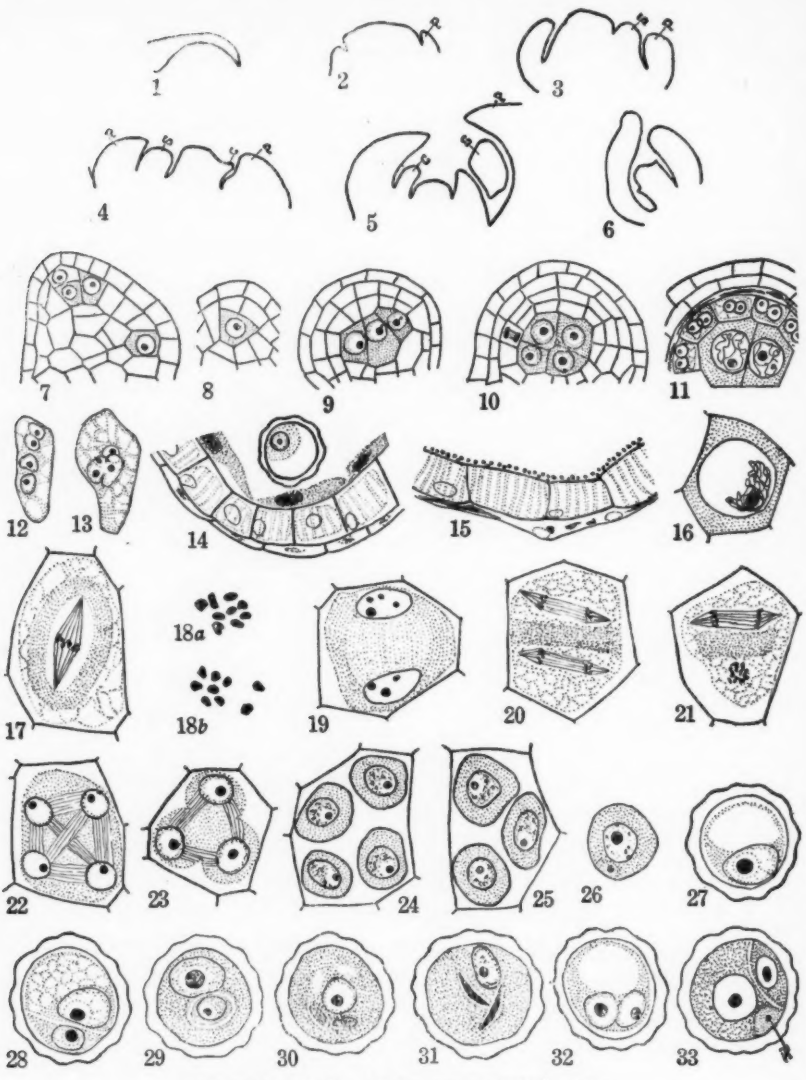
Microsporangium.—As the anther becomes four-lobed, in each lobe one or two hypodermal archesporial cells are differentiated (Fig. 7). The archesporial cells cut off the primary parietal cells on the outer side (Fig. 7). They divide anticlinally first (Fig. 8) and then periclinally to form two layers (Fig. 9). The outer one forms endothecium and the inner divides to give rise to ephemeral middle layer and the tapetum (Figs. 10 and 11). The archesporial cells after cutting off the primary parietal cells become sporogenous. They divide, enlarge and form pollen mother cells.

The tapetal cells are uninucleate but through mitotic divisions become two- to four-nucleate (Figs. 11–12). The nuclei fuse to form a single multinucleolate nucleus (Fig. 13). Four-nucleate condition of tapetal cells has been observed in *Beta vulgaris* (Artschwager, 1927), *Atriplex hymenelytra* (Billings, 1934), *Chenopodium album* (Bhargava, 1936) and *Suæda fruticosa* (Mahabale and Solanky, 1953).

After the formation of microspores, walls of the tapetal cells break down and protoplasts coalesce to form a continuous mass at the periphery of the pollen chamber (Fig. 14) which completely disappears later (Fig. 15). Tapetum is of the secretory type as in *Suæda fruticosa* (Mahabale and Solanky, 1953). The formation of a tapetal periplasmodium is not known in Centrospermales (Schnarf, 1931).

The endothecium becomes fibrous (Figs. 14 and 15). In mature anthers, small peculiar droplet-like bodies appear on the inner surface of the endothecium (Fig. 15). A few of the endothecial cells in the middle of each anther lobe do not get thickened. The epidermis degenerates (Fig. 14) and its remnants are found on the outer wall of the endothecium (Fig. 15).

Microsporogenesis.—The microspore mother cells undergo a period of rest and increase in size. The heterotypic division commences with thickening of reticulum threads (Fig. 11). The threads condense into a typical synizetic knot (Fig. 16). The metaphase spindle at the first division is surrounded by a clear space around which lie a dense cytoplasmic zone and a more vacuolated area (Fig. 17), as in *Chenopodium album* (Bhargava, 1936) and *Suæda fruticosa* (Mahabale and Solanky, 1953). Nine bivalents were counted at the metaphase (Figs. 18 *a* and 18 *b*). The interphase is well defined (Fig. 19). After a brief period of interkinesis the daughter nuclei enter the homotypic division. The spindles of this division may be parallel



TEXT-FIGS. 1–33. *Arthrocnemum indicum* Moq.

Figs. 1–6. Organogeny of the flower, × 132. P—perianth primordium; S—stamen primordium; C—carpel primordium. Figs. 7–11. Stages in the development of the anther, × 331. Fig. 12. A quadri-nucleate tapetal cell, × 705. Fig. 13. A tapetal cell showing fusion of nuclei, × 705. Fig. 14. Anther wall at microspore stage; note the degenerating tapetum, × 331. Fig. 15. Fibrous endothecium; note the degenerating epithelium, × 331. Figs. 16–22. Stages in the division of microspore-mother-cell, × 705. Figs. 18 *a*, 18 *b*. Metaphase

plates showing 9 bivalents. Fig. 23. Cytokinesis by furrowing, × 705. Figs. 24–25. Isobilateral and tetrahedral tetrads, × 705. Figs. 26–27. Uninucleate pollen grains, × 705. Figs. 28–29. Two-celled pollen grains, × 705. Figs. 30–31. Three-celled pollen grains, × 705. Fig. 32. A pollen grain with two vegetative nuclei, × 705. Fig. 33. A pollen grain with a prothallial cell—P.C., × 705.

(Fig. 20) or at right angles to each other (Fig. 21). Secondary spindles connect the four daughter nuclei with one another (Fig. 22).

The original wall of the microspore mother cells remain intact during the whole process of meiosis. Quadripartition occurs by furrowing (Fig. 23). The tetrads may be isobilateral (Fig. 24) or tetrahedral (Fig. 25). Microspores are liberated by the disintegration of the wall of the microspore mother cell.

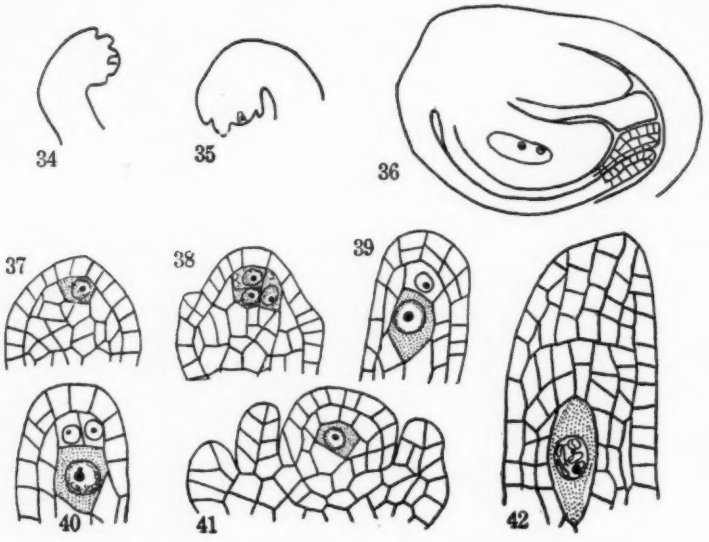
Male gametophyte.—The young microspore when separated from the tetrad has a prominent nucleus surrounded by dense cytoplasm (Fig. 26). As it increases in size its nucleus is pushed to the periphery by appearance of a large vacuole in the centre (Fig. 27). It develops exine with thickenings characteristic of the Chenopodiaceæ and thin intine (Fig. 27). The exine is perforated by oval pores arranged regularly all over.

The divisions of the microspore nucleus results in the formation of a large vegetative and small lenticular generative cell separated by a curved space (Fig. 28). The presence of a definite wall between the two as in some Chenopodiaceous plants (Cooper, 1935) was not noticed. The generative cell leaves its peripheral position and gets embedded in the cytoplasm of the tube cell (Fig. 29). It enlarges and divides to form two male cells which are oval first (Fig. 30), but become elongated and spindle-shaped later (Fig. 31). During all these stages the size of the pollen increases. The pollen grains are tri-cellular at the time of shedding as in *Spinaceæ oleracea* (Tuschnjakowa, 1929), *Chenopodium album* (Bhargava, 1936), and *Suæda fruticosa* (Mahabale and Solanky, 1953). Tri-nucleate pollen grains are characteristic of the whole order Centrospermales.

Abnormalities.—In some pollen grains the nucleus divides and gives rise to two vegetative nuclei (Fig. 32) as in *Chenopodium album* (Bhargava, 1936) and *Suæda fruticosa* (Mahabale and Solanky, 1953). In others there was a prothallial cell (Fig. 33, P.C.). Prothallial cell was also noticed in *Atriplex hymenelytra* by Billings (1934).

Megasporangium.—The crassinucleate bi-tegmic ovule arises as a blunt protuberance on the placenta. To begin with it is erect, but gradually curves (Fig. 34) and gets completely inverted (Fig. 35). Its curvature increases till it takes another turn through an angle of more than 90° (Fig. 36). The

two integuments arise exogenously, only epidermis taking part in their formation. These epidermal cells enlarge, project out, divide and establish apical cells (Fig. 38), which form two-layered integuments. The inner integument arises first followed by the outer one (Figs. 38 and 41). Only the outer



TEXT-FIGS. 34–42. *Arthrocnemum indicum* Moq.

Figs. 34–36. Curvature of ovule, × 147. Figs. 37–42. Megasporogenesis, × 475. Fig. 37. Hypodermal archesporial cell in the nucellus. Fig. 38. Multicellular archesporium. Fig. 39. Megaspore mother cell and primary parietal cell; note the integumentary apical cells. Fig. 41. L.S. Ovule showing two integuments. Fig. 42. L.S. Nucellus showing megaspore mother cell in early prophase and nucellar beak.

integument forms the micropyle (Fig. 36). The integuments are each two layered but the part of the inner integument forming the micropyle is thicker (Fig. 36) as in many families of Centrospermales (Maheshwari, 1945). Occasionally there is a prominent air space between the two integuments in the chalazal region. Such air space was also noticed in *Chenopodium album* (Bhargava, 1936) and *Suæda fruticosa* (Mahabale and Solanky, 1953).

The archesporium is differentiated as a single cell before the integuments (Fig. 37). Multicellular archesporium was also observed (Fig. 38). The archesporial cell divides to form a primary parietal cell and the megaspore mother cell (Fig. 38). The cutting off of a parietal cell was noticed in *Atriplex hymenelytra* (Billings, 1934), *Chenopodium album* (Bhargava, 1936),

Suæda fruticosa (Mahabale and Solanky, 1953), and some other plants of the Centrospermales.

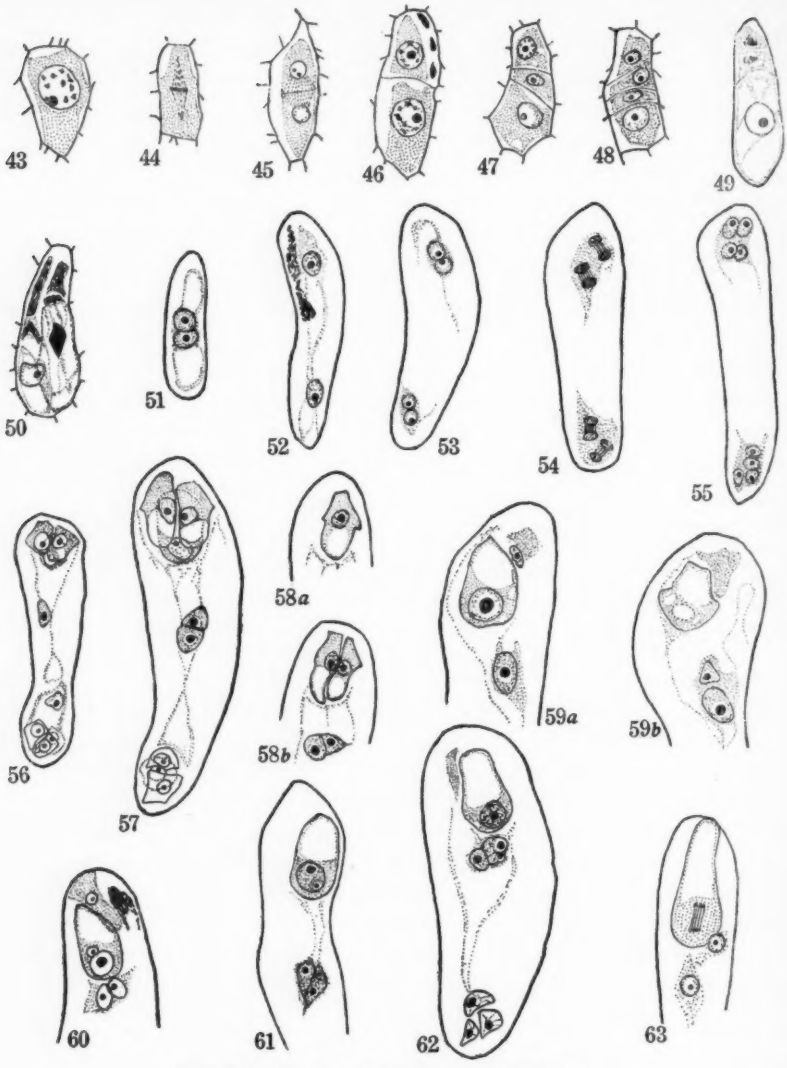
The primary parietal cell divides anticlinally first (Fig. 40) and then periclinally to form a few layers. The epidermis also divides to form nucellar beak (Figs. 39 and 42). The nucellus becomes massive, and the megaspore mother cell gets deep-seated (Fig. 42). The cells of the nucellar beak which are situated directly below the micropyle become narrow and radially elongated as in *Beta vulgaris* (Artschwager and Starrett, 1933), *Chenopodium album* (Bhargava, 1936) and *Suæda fruticosa* (Mahabale and Solanky, 1953).

Megasporogenesis.—The megaspore mother cell undergoes a long period of rest and enlarges (Figs. 39 and 40). It divides meiotically (Figs. 42–45) to form two dyad cells of which the micropylar one is smaller than the chalazal one (Fig. 46). Usually only chalazal dyad cell divides, resulting in a row of three cells (Fig. 47) or rarely both the dyad cells divide again to give rise to a linear tetrad of four megaspores (Fig. 48). Three to four megaspores were observed in *Basella* and *Ullucus* (Rocen, 1927), and three in *Atriplex* (Billings, 1934).

At times two archesporial cells become functional and give rise to double uninucleate embryo-sacs, of which one always degenerates (Figs. 50 and 52) and the other develops.

Embryo-sac.—The chalazal megaspore functions and gives rise to the embryo-sac. The nucleus is located in the centre and there are large vacuoles at both the ends of it in the long direction of the cell (Figs. 49–50). The two daughter nuclei formed after the first division (Fig. 51) reach their respective poles (Fig. 52), and divide again to form two nuclei at each pole (Fig. 53). They further divide (Fig. 54) and form octo-nucleate embryo-sac (Fig. 55). The micropylar nuclei are larger than the chalazal ones. During all these stages the size of the embryo-sac increases, a large central vacuole is formed and the nuclei lie in the scanty protoplasm which collects mostly at poles. The mature embryo-sac is monosporic, eight-nucleate and falls under the *Polygonum* type (Maheshwari, 1950).

Female gametophyte.—From the micropylar group of four nuclei differentiates the egg apparatus and upper polar nucleus, while the chalazal group differentiates into lower polar nucleus and three antipodals (Figs. 56–57). A young synergid has dense cytoplasm without vacuoles and is without hooks (Fig. 56). It develops a prominent hook and a large, basal, vacuole later (Figs. 57–58) as in *Chenopodium album* (Bhargava, 1936) and *Suæda fruticosa* (Mahabale and Solanky, 1953). The egg is broadly flask-shaped.



TEXT-FIGS. 43–63. *Arthrocnemum indicum* Moq.

Figs. 43–48. Megasporogenesis, × 475.—Fig. 43. Megaspore mother cell in diakinesis. Fig. 44. Megaspore mother cell in metaphase. Fig. 45. The same in telophase. Fig. 46. Two dyad cells. Fig. 47. A row of three cells; note that the micropylar dyad cell has failed to divide. Fig. 48. Linear tetrad of four megaspores. Figs. 49–58. Development of embryo-sac.—Fig. 49. Uninucleate embryo-sac. Fig. 50. Double uninucleate embryo-sacs. Figs. 51–55. Two, four and eight-nucleate embryo-sacs. Fig. 56. Embryo-sac showing polar nuclei

migrating towards centre. Fig. 57. The same fully formed. Figs. 58 *a*–58*b*. Hooked synergids and synergid-like egg. Figs. 59–62. Stages in fertilization, × 475.—Figs. 59 *a*–59 *b*. Discharge of male gametes from the pollen tubes. Fig. 60. One male gamete fusing with the egg and the other lying in the pollen tube. Fig. 61. Syngamy and triple fusion. Fig. 62. Triple fusion; note the antipodals. Fig. 63. Division of the zygote; note the free nuclei of the endosperm.

It has a large micropylar vacuole and its nucleus is embedded in the cytoplasm at the chalazal end (Figs. 57–63). The egg always extends beyond the synergids. In one case the egg has a large chalazal vacuole and appeared like a synergid (Fig. 58).

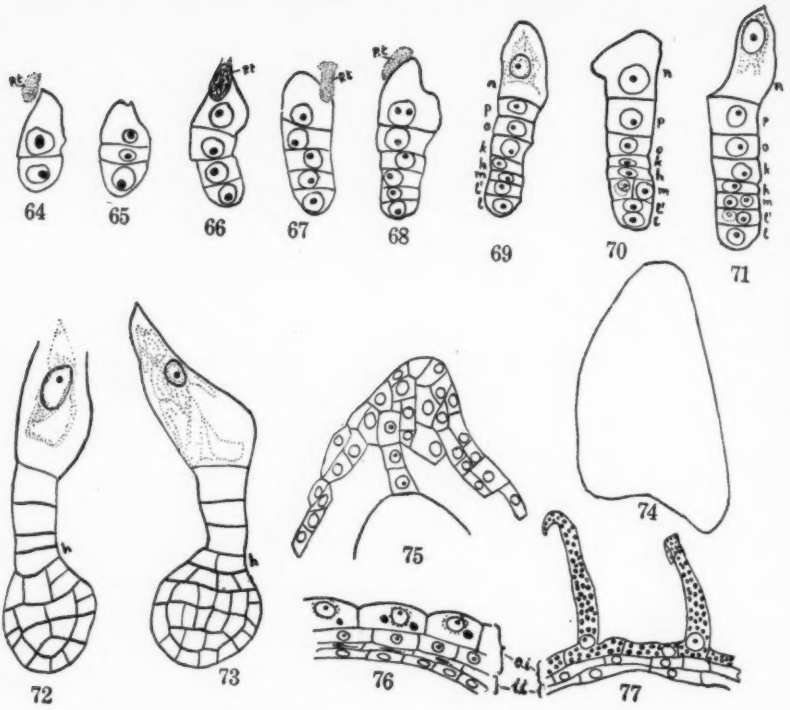
The polar nucleus from each pole migrates towards the centre of the embryo-sac (Fig. 56) where it meets its complement (Fig. 57). They enlarge in size but do not fuse before fertilization. The antipodals are seen upto the time of fertilization (Fig. 62), after which they degenerate. Usually they form a triangle, one above and two below (Figs. 56, 57, 62).

The embryo-sac when fully formed is spindle-shaped (Fig. 62). At first it is included in the micropylar end of the nucellus, but after fertilization it becomes elongated and curved by absorption of nucellar cells at the chalazal end.

Fertilization, endosperm and embryo.—The pollen tube enters the ovule through the micropyle and passing through the nucellar beak enters the embryo-sac. Here it enlarges and discharges the male cells (Figs. 59 *a* and 59 *b*). One male gamete fuses with the egg and the other with the two polar nuclei. Various stages in their fusion were observed (Figs. 59–62). The pollen tube is persistent and can be seen upto six-celled stage of pro-embryo (Figs. 66–68, *p.t.*).

The primary endosperm nucleus divides before the fertilized egg (Fig. 63). The endosperm is free nuclear, but later becomes cellular throughout the embryo-sac. The developing embryo absorbs greater part of the cellular endosperm and only a few layers of endosperm remain ensheathing the radicle of the embryo (Fig. 75) as in *Beta vulgaris* (Artschwager, 1927) and *Chenopodium album* (Bhargava, 1936).

The zygote undergoes a transverse division (Fig. 63) forming an apical and a basal cell (Fig. 64). The apical cell divides to form a row of three cells (Fig. 65); or both the cells divide simultaneously to form a four-celled pro-embryo (Fig. 66). By further divisions eight-celled pro-embryo is formed (Figs. 67–70). The cells can be designated from below upwards as *l*, *l'*, *m*, *h*, *k*, *o*, *p*, *n*. The embryo arises from *l*, *l'*, *m* and *h*. *k*, *o*, *p* form uniseriate suspensor while *n* enlarges, pushes itself in the nucellus at the micropylar end, and forms a haustorial cell (Figs. 71–73). Haustorial cell was



TEXT-FIGS. 64–77. *Arthrocnemum indicum* Moq.

Figs. 64–75. Stages in the development of embryo, × 311; note the development of the haustorial cell in Figs. 68–73 and the cellular endosperm in Fig. 75. Figs. 76–77. Development of the testa, × 220.

also observed in *Beta vulgaris* by Oksijuk (1927). At seven- or eight-celled stage of the pro-embryo *m* divides by a vertical wall and is followed by a vertical partition in *l'* (Fig. 71). The division of *l* lags behind. Dermatogen, periblem and pleurome are differentiated later (Figs. 72, 73). Division of *h* takes place very late (Fig. 73). Fig. 74 shows embryo with two cotyledons marked out. The suspensor remains uniseriate (Fig. 75). Uniseriate suspensor is also present in *Kochia scoparia* (Williams, 1932), while in *Chenopodium Bonus Henricus* (Soueges, 1920), *Beta vulgaris* (Artschwager, 1927), *Chenopodium album* (Bhargava, 1936) and *Suæda fruticosa* (Mahabale and Solanky, 1953) it is massive. Mature embryo is spiral as in many Centrospermales.

Seed coat.—The cells of both integuments are alike but later undergo some changes. In the cells of the outer layer of the outer integument large

taniferous granules are deposited. They bulge out and develop into unicellular hook-like hairs. The cells of the outer layer of the inner integument gradually disappear. The inner layers of the outer and inner integuments persist (Figs. 76–77, *i.i.*, *o.i.*).

Summary

1. The embryology of *Arthrocnemum indicum* Moq., a mangrove was studied. The floral parts develop in acropetal succession.

2. Archesporium is hypodermal in origin. The endothecium become fibrous. Middle layer is ephemeral. Tapetum is of the secretory type. The tapetal cells become bi-quadri-nucleate. Their nuclei fuse.

3. Nine bivalents were counted for the first time. Cytokinesis is by furrowing. The pollen-mother-cell-wall dissolves after the formation of microspores.

4. The reproductive cell is separated from the tube cell by a distinct space. The pollen grains are tri-cellular at the time of shedding. Some pollen grains had two vegetative nuclei while some formed a prothallial cell.

5. The ovule has two integuments, each one of which is two-layered. The inner only forms the micropyle and is thicker at the tip. An air space is present between the two integuments at the chalazal end. The nucellar epidermis and primary wall cells divide to form massive nucellus.

6. The archesporial cell in the nucellus is hypodermal and cuts off a primary parietal cell. Multicellular archesporium was also observed. Usually the division of the micropylar dyad cell is suppressed. Rarely tetrads of four megaspores are formed.

7. The chalazal megaspore is functional. The embryo-sac is monosporic and of *Polygonum* type. Synergids are hooked. Egg is flask-shaped and hangs lower than the synergids.

8. Various stages in syngamy and triple fusion were observed.

9. Endosperm is free nuclear but becomes cellular later throughout the embryo-sac. In mature seed only some layers of endosperm remain just above the radicle.

10. Development of the embryo was worked out. The suspensor is uniseriate. A single large haustorial cell is present at the base of the suspensor.

11. Testa has three layers of cells. The outer layer of the outer integument forms unicellular hairs.

In conclusion, we have to thank Prof. P. Maheshwari for some suggestions and literature.

LITERATURE CITED

1. Artschwager, E. .. "Development of flower and seed in sugar beet," *J. Agri. Res.*, 1927, **34**, 1–25.
2. ——— and Starrett, R. C. "The time factor in fertilization and embryo development in the sugar beet," *Ibid.*, 1933, **47**.
3. Bhargava, H. R. .. "The life-history of *Chenopodium album* Linn.," *Proc. Ind. Acad. Sci.*, 1936, **4B**, 179–200.
4. Billings, F. H. .. "Male gametophyte of *Atriplex hymenelytra*," *Bot. Gaz.*, 1934, **95**, 477–84.
5. Cooper, G. O. .. "Cytological studies in the Chenopodiaceæ, 1. Microsporogenesis and pollen development," *Ibid.*, 1935, **97**, 169–78.
6. Mahabale, T. S. and Solanky, I. N. .. "Studies in the Chenopodiaceæ, 1. Embryology of *Suæda fruticosa* Forsk." (In press), 1953.
7. Maheshwari, P. .. "Place of Angiosperm embryology in research and teaching," *Jour. Ind. Bot. Soc.*, 1945, **24**, 25–41.
8. ——— .. *An Introduction to the Embryology of Angiosperms*, 1950, McGraw Hill Book Co., New York.
9. Oksijuk, P. .. "Entwicklungsgeschichte der Zuckerrube *Beta vulgaris*," *Bull. Jard. Bot. Kieff*, 1927, **5–6**, 1–19 (In Russian with a summary in German).
10. Rocen, T. .. "Zur Embryologie der Centrospermen," Dis. Uppasala," 1927.
11. Schnarf, K. .. *Embryologie der Angiospermen*, Berlin, 1927.
12. ——— .. *Vergleichende Embryologie der Angiospermen*, Berlin, 1931.
13. Souègès, R. .. "Development de l'embryon chez le *Chenopodium Bonus Henricus* L.," *Bull. Soc. Bot. France*, 1920, **67**, 233–57.
14. Tuschnjakowa, M. .. Untersuchungen uber die Kernbeschaffenheit einiger diozischer Pflanzen," *Flanta*, 1929, **7**, 427–43.
15. Williams, M. E. .. "The Development of Embryo of *Kochia scoparia*." *Bull. Torrey Bot. Club*, 1932, **59**, 391–400.

793–54 Printed at The Bangalore Press, Bangalore City, by C. Vasudeva Rao, Superintendent and Published by The Indian Academy of Sciences, Bangalore

EFFECT OF FLUORINE ON THE COMPOSITION OF BONES: CHANGES IN THE COMPOSITION OF BONES OF RATS—PART I

By T. K. Wadhwani

(*Section of Pharmacology, Indian Institute of Science, Bangalore-3*)

Received December 8, 1953

(Communicated by Dr. M. K. Subramaniam, D.Sc., F.A.Sc.)

Introduction

The inevitable result of fluorine ingestion is its concentration in bones. Other changes in the composition of bones have been found to be different by different workers.[1-11] This is mainly due to the fact that the effect of fluorine on the composition of bones is not invariant, but is determined by the quantity of fluorine ingested, the composition of the diet fed, the age and the species of the experimental animal. Some of the different results reported in the literature by different workers may thus be assumed to be due to such differences in the methods of experimentation.

Besides such differences in the methods of experimentation, in the studies, carried out hitherto, on the effect of fluorine on the composition of bones, the following two points seem to have been ignored or overlooked. Firstly, despite the data that fluorine reduces the availability of calcium, phosphorus and nitrogen for the system by decreasing the food intake of the animal, and by interfering with the absorption and retention of these elements, no attempt has been made to create the similar conditions of sub-normal nutrition in the control animals, and thus seek to distinguish the effect of fluorine on the development of bones as due to such conditions from the other effects of fluorine. Secondly, whenever the question of the interference or the role of any element in the development of bones is sought to be studied, it has been common practice to investigate the mineral portion only of the bone. Bone is composed of inorganic as well as organic matter. The inorganic matter consists of mineral salts, chiefly the phosphate of lime. The organic matter is white fibrous tissue which consists of fine fibres united by ground substance into inter-lacing bundles, and connective tissue cells placed in rows among the bundles of fibres. By the biochemical study of bone growth, Hammett[12] has shown that the mineral complement of bone, under normal and similar conditions of experimentation, at any particular age of the rat, is fairly constant, not only in regard to its total percentage but also in regard

to the percentages of calcium, phosphorus and magnesium. If the percentage of mineral matter of bone is constant under a certain set of conditions, there is no reason as to why it should not be assumed that the organic portion of the bone, the white fibrous tissue, should also be constant under a similar set of conditions. For the normal development of bone, it is essential that not only the normal quantities of lime salt should be deposited in the ground substance but that also the normal quantity of fibrous tissue, the supporting collagen, should be formed. Under such circumstances, there seems to be no justification as to why the organic matter of bone has not been taken into account in the studies on the development of bone in normal as well as in pathological states. For the proper understanding of the formation of bones, it is necessary to study both the processes, one concerning the formation of the supporting collagen, and the other, the deposition of calcium phosphates. This is particularly important for the study of the effect of fluorine on bone, as it has been found that, as a result of fluorine ingestion, the bones are weak and fragile, and that the fractures of the bones are a common occurrence in humans and cattle in the areas of endemic fluoriosis. It is to the fibrous tissue that the bones owe their toughness and elasticity.

Bearing these considerations in mind, studies have been carried out to investigate the effect of fluorine on the composition of bones. The information has been sought on the effect of fluorine on the nitrogen, calcium, magnesium, phosphorus, carbonate, sodium and, in some cases, potassium content of bones, and as it is influenced by the quantity of sodium fluoride administered, the composition of the diet fed, and the conditions of partial starvation produced as a result of fluorine ingestion.

GENERAL PLAN OF THE EXPERIMENT

The experiments have been carried out on white rats, 5 to 6 weeks old. The rats have been divided into groups to minimise the variations due to sex, age, weight and litter origin. For the study of the effect of the conditions of semi-starvation, produced as a result of fluorine ingestion, on the composition of bones, the individual control method of paired-feeding has been employed. Five pairs of rats have been used for each experiment, each pair comprising of rats of same sex, weight and, if possible, of same litter origin, and the food intake has been equalised within pairs. To another set of five rats of same sex and weight, the same diet has been given *ad libitum*.

The diet consisted of cotton seed globulin 9%, gelatin 4%, nitrogen-free starch 65%, salts 4%, choline hydrochloride in sugar (1 : 9) 1%, cystine in

sugar (1 : 19) 1%, vegetable fat 16%, and the necessary amounts of vitamins A, D and of B complex. The other diets used in these investigations have been prepared from this diet by eliminating from it that constituent or those constituents whose influence on the composition of bones, under the conditions of fluorine poisoning, is sought to be studied.

The quantity of fluorine administered was 2 mgms. of NaF per day per rat. The quantity was doubled in cases where the effect of larger concentration of fluorine on the composition of bones was studied. In every case, sodium fluoride in solution was given per os, delivered from a 1 c.c. graduated pipette, few hours before the food was offered. The reasons for giving the dose dissolved in water and not admixed with food, and for giving it before the food is offered have been explained in another communication.[13]

Selection of Bones for Analysis

In all studies on bone development under different physiological conditions, it has been the general practice to analyse one or two bones, whose choice has been a matter of individual preference, and assume the results as applicable to the whole skeletal system.[3, 4, 5, 6] The results obtained by such an arbitrary selection of bones have been variable sometimes.[3, 5] Besides this variation observed in the effect of fluorine on the development of different bones, Hammett[12] has shown that normally there are significant differences in the rates of calcification of the humerus and femur of rat. With the aid of the radio active isotope of phosphorus, Hevesy *et al.*[14] have observed that, among the calcified tissues, the scapulas and the epiphyses are the most active, and therefore best indicative of the changes occurring in the system. Based on such observations, and the likelihood that the changes in the composition of bones, as a result of fluorine ingestion, might not be of a drastic nature, it was considered desirable to analyse as many bones as possible. The following bones were selected for the analysis of their constituents: ribs, thoracic vertebræ, fibula, radius, ulna, humerus, scapula, femur, tibia and epiphyses separated from femur and tibia.

At the end of the experimental period, which was decided in the light of the earlier work on the effect of fluorine on the metabolism of nitrogen, calcium and phosphorus,[13] the animals were killed with chloroform. The skin was removed, and the skeleton was separated from the accompanying flesh by the method of Subrahmanyan, Duckworth and Godden.[15] The bones were defatted first with absolute alcohol, and then with ether. Except the long bones, the fat and water-free bones were dried in a desiccator, weighed and powdered. The long bones were cut open longitudinally and the dried marrow was removed as far as possible. The fat, water and

marrow free bones were then dried in a desiccator, weighed and powdered. The various elements in the powdered bones were then estimated by the procedure outlined below.

ANALYTICAL METHODS

Nitrogen was estimated by the usual micro-Kjeldahl method, and carbonate, by the method of Van Slyke and Folch.[16] After the carbonate was estimated in the bone sample, the combustion tube, containing the dissolved bone salt, was taken out from the Van Slyke apparatus, and was immersed in a boiling water-bath for the complete dissolution of the bone salt. The solution was then cooled, filtered and made up to volume. Calcium was estimated in a suitable aliquot of this solution by the usual permanganate titration method, precipitating it at pH 3 to prevent the coprecipitation of magnesium oxalate, calcium phosphate and reducing substances. The clear supernatant of the calcium oxalate precipitate was then analysed for magnesium by precipitating it as magnesium ammonium phosphate, and estimating it as phosphate by the method of Fiske and Subba Rao,[17] using a photo-electric colorimeter. Phosphate of the solution was estimated by the same method. For this, a small aliquot of the solution was first digested with perchloric acid to oxidise the protein matter that comes into the solution from the bone. The oxidation of this protein matter, prior to the estimation of phosphorus, is essential, as it precipitates with the acid ammonium molybdate reagent when it is added for reaction with phosphate. This results in turbid solutions, and consequently in higher phosphate values. For the estimation of sodium, another aliquot of the solution was taken in a centrifuge tube, and was made slightly alkaline with ammonia, whereby the bulk of calcium phosphate was precipitated. This was separated from the solution by centrifugation. The clear supernatant together with the clear washings of the calcium phosphate precipitate was transferred to another centrifuge tube, and evaporated to dryness in an oven at 110° C. Sodium was estimated in the dry residue by the method of Darnell and Walker.[18] Potassium was estimated in an aliquot of the hydrochloric acid extract of bone by the method of Looney and Dyer.[19] The values of potassium for different bones, except those for the exostoses reported in the second part of this piece of investigation, are not included in the results presented, as sometimes highly erratic values have been obtained.

PRESENTATION OF RESULTS

The results are expressed in percentages on the basis of water, fat and marrow free bone. For the per cent. of carbonate in the bone, the corresponding per cent. of calcium has been calculated. This has been deducted

from the total per cent. of calcium in the bone, and the ratio of residual calcium to phosphate has been calculated. The values of fluorine in the bone have not been included in the data presented. These will be presented and discussed separately in another communication.

When sodium fluoride has been administered at the rate of 2 mgm. per day, per rat, for 14 weeks, it is seen (Table I) that there are no significant variations in the percentages of calcium, phosphorus and magnesium between the bones of the experimental rat and the corresponding bones of its individual control which has been given the same quantity of feed as that consumed by the experimental rat. Comparatively, the bones of the normal rats, which have been given the same diet *ad libitum*, contain higher percentages of calcium and phosphorus. It has been shown earlier[13] that, as a result of fluorine ingestion, the food intake of the animal is markedly reduced, and consequently the supply of the essential constituents in the system is decreased. As the food intake is equalised between the experimental and control animals, the latter receive less of the essential constituents than the normal rats receiving the diet *ad libitum*, and naturally show lesser calcification of the bones than the normal animals. Despite the fact that the experimental animals retain less of calcium and phosphorus than their individual controls,[13] the bones of the experimental and control rats have more or less the same percentages of calcium and phosphorus. In other words, this would mean that, for the same quantity of calcium and phosphorus retained in the system, the experimental rats deposit more of these elements in the bones than the controls.

The bones of the experimental rats contain less nitrogen, more carbonate and sodium than the corresponding bones of the control and normal animals.

There is no consistent or significant variation in the Ca : P ratio of different bones from experimental, control and normal animals.

Qualitatively, similar results have been obtained in the case of bones of rats, receiving 4 mgm. of NaF per day, per rat, for 10 weeks (Table II). The bones of the experimental rats contain less nitrogen, more carbonate and sodium than the corresponding bones from control and normal animals. The bones of the normal rats contain higher percentages of calcium and phosphorus than the bones of the experimental and control animals. As in the previous case, so in this, the experimental rats, despite the diminished retention of calcium and phosphorus,[13] have higher percentages of these elements in the bones than the controls. In the case of rats receiving 2 mgm. of NaF per day, per rat, the percentages of calcium and phosphorus in the bones of the experimental and control rats are seemingly similar. It is only

TABLE I

The effect of fluorine on the composition of the bones when fluorine as sodium fluoride has been given at the rate of 2 mgm. per day, per rat, for 14 weeks

Composition of the diet fed: N=2·20%; Ca=0·384%; P=0·27%; F=0·00045%.
The results are expressed in per cents. on the basis of water, fat and marrow free bone.

		Nitrogen	Carbonate	Calcium	Calcium corresponding to carbonate	Residual calcium	Phosphorus	Ca : P ratio	Magnesium	Sodium
Ribs	E	3·94	5·08	24·27	3·39	20·88	10·64	1·96	0·54	0·40
	C	4·78	4·47	24·22	2·98	21·24	11·19	1·89	0·52	0·36
	N	4·98	4·27	24·52	2·84	21·68	11·35	1·91	0·50	0·36
Spine	E	3·78	4·83	23·75	3·22	20·53	10·55	1·94	0·43	0·38
	C	5·00	4·24	23·63	2·83	20·80	10·75	1·93	0·49	0·28
	N	5·02	4·34	25·25	2·89	22·36	11·90	1·88	0·46	0·30
Fibula	E	3·77	5·39	24·17	3·59	20·58	10·65	1·93	0·42	0·42
	C	5·50	4·93	24·43	3·28	21·15	10·95	1·93	0·44	0·29
	N	5·45	4·54	25·40	3·03	22·37	11·85	1·89	0·44	0·32
Radius	E	3·95	5·19	23·88	3·46	20·42	11·02	1·85	0·49	0·42
	C	4·35	3·80	22·78	2·53	20·25	11·22	1·80	0·49	0·32
	N	4·97	4·10	25·75	2·73	23·02	12·02	1·91	0·45	0·34
Ulna	E	3·73	5·20	24·28	3·46	20·82	11·67	1·78	0·48	0·43
	C	4·85	4·72	24·01	3·15	20·86	11·29	1·84	0·45	0·33
	N	5·02	4·45	25·95	2·96	22·99	11·94	1·92	0·45	0·34
Humerus	E	3·55	4·76	25·27	3·17	22·10	11·54	1·91	0·53	0·45
	C	5·36	4·12	25·12	2·74	22·38	11·74	1·90	0·50	0·36
	N	5·28	4·25	26·60	2·83	23·77	12·38	1·92	0·47	0·35
Scapula	E	4·30	4·84	22·42	3·22	19·20	10·52	1·82	0·42	0·39
	C	5·79	4·35	21·81	2·90	18·91	10·04	1·88	0·52	0·31
	N	5·98	4·40	23·20	2·93	20·27	10·78	1·88	0·39	0·29
Femur	E	3·45	5·13	24·64	3·42	21·22	11·38	1·86	0·49	0·47
	C	4·16	4·44	24·67	2·96	21·71	11·22	1·93	0·49	0·38
	N	4·88	4·10	26·54	2·73	23·81	12·50	1·90	0·45	0·35
Tibia	E	3·55	4·97	24·92	3·31	21·61	11·51	1·88	0·45	0·47
	C	4·51	4·30	24·72	2·87	21·85	11·24	1·94	0·51	0·34
	N	4·94	4·05	25·20	2·70	23·50	12·12	1·94	0·47	0·35
Epiphyses	E	4·64	4·47	21·17	2·98	18·19	9·41	1·93	0·49	0·40
	C	5·45	3·58	19·39	2·36	17·01	9·00	1·88	0·40	0·31
	N	5·95	3·28	22·87	2·19	20·68	10·90	1·90	0·39	0·30

E=Bones from experimental rats; C=Bones from control rats; N=Bones from normal rats.

The effect of fluorine on the composition of bones of rats when fluorine as sodium fluoride has been given at the rate of 4 mgm. per day, per rat, for 10 weeks

	C	5·45	3·58	19·39	2·36	17·01	9·00	1·88	0·40	0·31
	N	5·95	3·28	22·87	2·19	20·68	10·90	1·90	0·39	0·30

E=Bones from experimental rats; C=Bones from control rats; N=Bones from normal rats.

The effect of fluorine on the composition of bones of rats when fluorine as sodium fluoride has been given at the rate of 4 mgm. per day, per rat, for 10 weeks

Composition of the diet fed: N=2·20%; Ca=0·384%; P=0·27%; F=0·00045%.
The results are expressed in per cents. on the basis of water, fat and marrow free bone.

		Nitrogen	Carbonate	Calcium	Calcium corresponding to carbonate	Residual calcium	Phosphorus	Ca : P ratio	Magnesium	Sodium
Ribs	E	3·78	4·16	24·12	2·77	21·35	11·50	1·85	0·37	0·45
	C	4·80	2·38	22·72	1·58	21·14	11·50	1·84	0·78	0·34
	N	5·02	3·50	24·50	2·33	22·17	11·84	1·87	0·49	0·35
Spine	E	3·71	3·95	24·75	2·63	22·12	12·10	1·83	0·47	0·42
	C	4·83	2·47	22·95	1·64	21·31	11·42	1·86	0·73	0·29
	N	5·23	3·02	24·98	2·01	22·97	11·92	1·93	0·47	0·30
Fibula	E	3·28	4·94	25·27	3·29	21·98	11·88	1·85	0·44	0·46
	C	4·42	2·94	23·75	1·96	21·79	11·82	1·84	0·65	0·33
	N	5·40	3·58	25·57	2·38	23·19	12·10	1·91	0·44	0·33
Radius	E	3·40	4·89	24·10	3·26	20·84	11·23	1·85	0·28	0·46
	C	4·48	3·24	22·52	2·16	20·36	11·45	1·78	0·66	0·33
	N	5·22	3·46	25·20	2·30	22·90	11·98	1·91	0·45	0·34
Ulna	E	3·41	4·92	24·75	3·28	21·47	11·75	1·82	0·39	0·48
	C	4·50	2·89	23·50	1·92	21·58	11·25	1·92	0·61	0·33
	N	5·32	3·57	25·52	2·38	23·14	12·35	1·87	0·47	0·34
Humerus	E	3·35	4·99	24·95	3·32	21·63	11·23	1·92	0·45	0·51
	C	5·67	2·62	22·25	1·75	20·50	10·98	1·86	0·70	0·35
	N	5·75	3·87	26·15	2·58	23·57	12·67	1·86	0·48	0·36
Scapula	E	3·52	3·96	21·95	2·64	19·31	10·27	1·88	0·43	0·42
	C	5·79	2·26	19·89	1·50	18·39	10·02	1·83	0·67	0·28
	N	6·20	2·85	22·98	1·90	21·08	11·12	1·89	0·38	0·28
Femur	E	3·18	4·53	25·21	3·02	22·19	11·87	1·87	0·43	0·50
	C	4·75	3·23	23·75	2·15	21·60	11·23	1·92	0·67	0·32
	N	5·12	3·73	26·52	2·48	24·04	12·82	1·87	0·47	0·34
Tibia	E	3·28	4·87	25·50	3·25	22·25	12·00	1·85	0·48	0·51
	C	4·51	2·89	23·45	1·93	21·52	11·27	1·91	0·68	0·33
	N	5·24	3·67	26·85	2·44	24·41	12·72	1·92	0·46	0·36
Epiphyses	E	3·50	3·99	22·30	2·66	19·64	10·75	1·82	0·41	0·42
	C	5·61	2·81	19·80	1·87	17·93	9·37	1·91	0·49	0·29
	N	6·20	2·85	23·02	1·90	21·12	11·02	1·91	0·38	0·31

E=Bones from experimental rats; C=Bones from control rats; N=Bones from normal rats.

when it is considered that the experimental rats have retained less of calcium and phosphorus than the control animals, that the fact emerges that, for the same quantity of these elements retained, the experimental animals have deposited more of these elements in the bones than the controls. In the case of rats receiving 4 mgm. of NaF per day, per rat, the differences in the percentages of calcium deposited in the bones of experimental and control rats are quite marked, whereas the differences in the percentages of phosphorus are not so marked and uniform. As the high percentage of calcium in the bones of the experimental rats is partly counter-balanced by the high percentage of carbonate, the ratio, residual Ca : P, is not significantly different from the similar ratio for the bones of control and normal animals. Unlike the bones of the rats in the previous experiment (Table I), the bones of the control animals contain higher percentages of magnesium than the corresponding bones of the experimental and normal animals.

The strange fact, that the experimental animals, despite their lower retention of calcium and phosphorus, have higher percentages of these elements in the bones than the controls, may be explained in the following two ways. Firstly, probably, in fluorine poisoning, the equilibria among the different fractions of calcium and among the different fractions of phosphorus are so disturbed as to cause an increase in the ionic concentration of calcium and phosphorus to an extent that the solubility product of calcium phosphate is exceeded, which is thus precipitated, resulting in its deposition in larger quantity in the experimental bones than in the control bones. Secondly, as a result of fluorine ingestion, the food intake and the general metabolic rate of the animal are reduced.[13] The reduced food intake may be sufficient for the reduced metabolism of the experimental animal, but, when given to the normal animal with normal metabolic rate, it will cause a state of sub-normal nutrition, which may be reflected in the diminished calcification of bones. The increased percentages of calcium and phosphorus in the bones of the experimental rats, however, may be due to both these causes. The decreased percentage of nitrogen in the bones of the experimental rats would suggest that fluorine either interferes with the formation of the protein matter in bone or causes its greater destruction or that it does both.

Further studies have been carried out to confirm and clarify the results presented above, and to find out the effect of the dietary constituents on the toxic action of fluorine on the composition of bones. The rats have been placed on diet (i) deficient in nitrogen, for 4 weeks; (ii) deficient in calcium and phosphorus, for 4 weeks; and (iii) deficient in nitrogen, calcium and phosphorus, for 3 weeks, and in each case, have been given 2 mgm. of NaF per day, per rat. The results are given in Tables III, IV and V.

TABLE III

The effect of fluorine on the composition of bones of rats placed on diet deficient in nitrogen, when fluorine as sodium fluoride has been given at the rate of 2 mgm. per day, per rat, for 4 weeks

Composition of the diet fed: N=0·0152%; Ca=0·384%; P=0·27%; F=0·00045%.
The results are expressed in per cents. on the basis of water, fat and marrow free bone.

		Nitrogen	Carbonate	Calcium	Calcium corresponding to carbonate	Residual calcium	Phosphorus	Ca : P ratio	Magnesium	Sodium
Ribs	E	4·73	4·61	23·34	3·07	20·27	10·98	1·84	0·60	0·28
	C	5·09	3·73	22·81	2·49	20·32	11·18	1·82	0·60	0·25
Spine	E	4·45	3·46	21·13	2·30	18·83	10·58	1·78	0·50	0·25
	C	5·05	3·43	21·59	2·29	19·30	10·91	1·77	0·52	0·23
Fibula	E	3·58	3·97	19·36	2·65	16·71	8·97	1·86	0·52	0·29
	C	4·30	3·75	20·84	2·50	18·34	10·47	1·75	0·54	0·27
Radius	E	4·48	3·95	23·33	2·63	20·70	11·03	1·87	0·48	0·31
	C	5·18	3·60	22·51	2·40	20·11	10·71	1·87	0·53	0·28
Ulna	E	3·99	3·83	23·29	2·55	20·74	10·91	1·90	0·48	0·30
	C	4·48	3·83	23·29	2·55	20·74	11·11	1·86	0·48	0·28
Humerus	E	4·00	3·86	23·52	2·57	20·95	11·50	1·82	0·55	0·33
	C	4·28	3·33	22·62	2·22	20·40	10·91	1·87	0·52	0·30
Scapula	E	4·58	2·99	20·54	1·99	18·55	9·80	1·89	0·49	0·25
	C	5·14	2·18	19·89	1·45	18·44	9·61	1·92	0·53	0·22
Femur	E	3·97	3·91	23·68	2·61	21·07	11·67	1·80	0·50	0·33
	C	4·41	3·74	23·34	2·49	20·85	11·81	1·76	0·47	0·31
Tibia	E	3·60	3·70	21·54	2·46	19·08	10·35	1·84	0·42	0·33
	C	3·93	3·40	22·55	2·32	20·33	11·23	1·80	0·45	0·30
Epiphyses	E	5·99	2·73	16·68	1·82	14·86	7·61	1·95	0·36	0·30
	C	6·75	2·67	17·98	1·78	16·20	9·16	1·77	0·47	0·22

E=Bones from experimental rats. C=Bones from control rats.

TABLE IV

The effect of fluorine on the composition of bones of rats placed on diet deficient in calcium and phosphorus, when fluorine as sodium fluoride has been given at the rate of 2 mgm. per day, per rat, for 4 weeks

Composition of the diet fed: N=1·8282%; Ca=0·00536%; P=0·0085%; F=0·000761%.
The results are expressed in per cents. on the basis of water, fat and marrow free bone.

		Nitrogen	Carbonate	Calcium	Calcium corresponding to carbonate	Residual calcium	Phosphorus	Ca : P ratio	Magnesium	Sodium
Ribs	E	5·11	3·83	18·44	2·55	15·89	8·72	1·82	0·34	0·30
	C	5·53	3·12	18·90	2·08	16·82	8·89	1·90	0·35	0·24
Spine	E	5·06	3·93	19·28	2·62	16·66	9·21	1·81	0·33	0·27
	C	5·17	2·40	18·50	1·60	16·90	9·43	1·79	0·35	0·23
Fibula	E	4·45	3·23	20·72	2·15	18·57	10·78	1·72	0·48	0·37
	C	5·52	1·97	19·92	1·31	18·61	10·81	1·71	0·51	0·30
Radius	E	4·21	3·54	20·75	2·36	18·39	10·58	1·74	0·52	0·36
	C	5·21	2·12	18·63	1·41	17·22	9·48	1·81	0·55	0·30
Ulna	E	3·75	3·62	21·21	2·41	18·80	10·80	1·74	0·51	0·36
	C	5·53	2·12	19·73	1·41	18·32	10·72	1·71	0·52	0·31
Humerus	E	3·54	3·89	20·56	2·59	17·97	10·16	1·77	0·49	0·40
	C	5·95	2·23	18·57	1·48	17·09	9·73	1·75	0·52	0·33
Scapula	E	4·75	3·57	18·01	2·38	15·63	8·49	1·84	0·46	0·32
	C	6·35	2·31	12·09	1·54	10·55	6·20	1·70	0·57	0·25
Femur	E	3·75	3·75	20·42	2·50	17·92	10·11	1·77	0·52	0·41
	C	5·20	2·67	15·96	1·78	14·18	7·57	1·87	0·60	0·33
Tibia	E	3·67	3·63	17·26	2·42	14·84	8·30	1·79	0·45	0·42
	C	5·32	2·47	15·39	1·65	13·74	7·68	1·79	0·52	0·31
Epiphyses	E	4·38	3·27	17·56	2·18	15·38	8·44	1·82	0·50	0·31
	C	6·10	2·06	15·69	1·37	14·32	7·83	1·83	0·62	0·25

E=Bones from experimental rats. C=Bones from control rats.

TABLE V

The effect of fluorine on the composition of bones of rats placed on diet deficient in nitrogen, calcium and phosphorus, when fluorine as sodium fluoride has been given at the rate of 2 mgm. per day, per rat for 3 weeks

Composition of the diet fed: N=0·0157%; Ca=0·0044%; P=0·0038%; F=0·0003905%.
The results are expressed in percents on the basis of water, fat and marrow free bone.

		Nitrogen	Carbonate	Calcium	Calcium corresponding to carbonate	Residual calcium	Phosphorus	Ca : P ratio	Magnesium	Sodium
Ribs	E	3·05	3·99	20·67	2·66	18·01	10·09	1·78	0·51	0·35
	C	5·66	3·08	16·21	2·05	14·16	8·42	1·68	0·64	0·25
Spine	E	3·61	3·60	20·46	2·40	18·06	10·11	1·78	0·53	0·29
	C	5·23	2·25	17·38	1·50	15·88	8·92	1·78	0·67	0·22
Fibula	E	3·86	3·38	20·83	2·25	18·58	10·24	1·81	0·53	0·35
	C	6·07	2·15	17·21	1·43	15·78	8·88	1·77	0·73	0·29
Radius	E	3·67	3·72	20·83	2·48	18·35	10·53	1·74	0·50	0·40
	C	5·41	1·77	17·02	1·18	15·84	8·74	1·81	0·55	0·29
Ulna	E	3·58	3·81	21·74	2·54	19·20	10·15	1·89	0·47	0·42
	C	6·62	2·22	16·97	1·48	15·49	8·42	1·84	0·74	0·30
Humerus	E	3·27	4·01	23·19	2·68	20·51	11·04	1·85	0·43	0·41
	C	5·60	2·03	15·34	1·35	13·99	6·96	2·01	0·61	0·31
Scapula	E	3·10	3·70	20·01	2·47	17·54	10·09	1·74	0·48	0·31
	C	5·92	2·57	16·42	1·71	14·71	8·69	1·69	0·62	0·24
Femur	E	2·96	3·55	20·82	2·36	18·46	10·28	1·79	0·50	0·47
	C	4·85	2·33	18·78	1·55	17·23	9·60	1·79	0·56	0·32
Tibia	E	2·94	3·57	20·35	2·38	17·97	9·99	1·80	0·42	0·45
	C	4·93	2·07	17·95	1·38	16·57	8·92	1·86	0·55	0·31
Epiphyses	E	2·25	3·75	21·55	2·50	19·05	10·87	1·75	0·49	0·33
	C	6·16	2·71	15·85	1·81	14·04	7·37	1·90	0·64	0·23

E=Bones from experimental rats. C=Bones from control rats.

As the control rats have sometimes consumed less diet than the experimental ones, no data are given regarding the composition of the bones of the rats given the same diet *ad libitum*. From the data presented, it will be seen that the results obtained in the previous experiments (Tables I and II) are confirmed. The changes noticed in the composition of bones of the experimental rats (*vide* Tables I and II) are very distinctly indicated in the case of the rats placed for 3 weeks on diet deficient in nitrogen, calcium and phosphorus. When the diet is deficient in nitrogen or calcium and phosphorus, the effect of fluorine on that constituent of the bone is more marked than when the diet is normal in regard to that constituent. When the diet is normal with respect to its calcium and phosphorus content, and deficient in nitrogen, as a result of fluorine ingestion, the percentage of nitrogen in the bones is higher than that in the bones of the rats, placed on diet deficient not only in nitrogen but in calcium and phosphorus also. The presence of calcium and phosphorus in the diet seems to exert a protective action against the effect of fluorine on the composition and development of bones.

SUMMARY AND CONCLUSIONS

1. Studies have been carried out on the effect of fluorine on the composition of bones of rats. Though no consistent variations have been observed in the Ca : P ratio of fluorosis bones, these have been found to contain less nitrogen, and more calcium, phosphorus, carbonate and sodium than the corresponding bones of the controls. In rats receiving 2 mgm. of NaF per day for 14 weeks, the magnesium content of the bones has not been found to be altered, whereas, it has been observed to decrease in the case of rats receiving 4 mgm. of NaF per day for 10 weeks. 2. These results have been further confirmed in the rats, placed on diet deficient in (i) nitrogen, (ii) calcium and phosphorus, and (iii) nitrogen, calcium and phosphorus. 3. The changes in composition have been observed in all bones. Though in some cases, the changes in the composition of scapulas and epiphyses are the most marked, it cannot be definitely stated that, in fluorine poisoning, these bones are most affected. 4. Calcium and phosphorus of the diet have been found to exert a protective action against the effect of fluorine on the composition of bones. 5. The data presented in regard to the carbonate and magnesium content of the experimental bones are in direct contrast to the findings of the earlier workers.[1, 4, 9, 11]

The results are discussed in the second part of this piece of investigation.

The author wishes to thank Prof. V. Subrahmanyan and Dr. N. N. De for their kind help. The author's thanks are also due to the Trustees, Lady

Tata Memorial Trust, for the award of the scholarship for the period during which this work was carried out.

References

1. Forbes, E. B. *et al.* .. *Ohio Agric. Exp. Stat. Bull.*, 1921, No. 347.
2. Bethke, R. M., Kick, C. H., Edgington, B. H. and Wilder, O H. *Am. Soc. Anim. Pro. Proc.*, 1929, 29. [Quoted from McClure, 1933.]
3. McClure, F. J. and Mitchell, H. H. .. *J. Biol. Chem.*, 1931, **90**, 297.
4. Kick, C. H., Bethke, R. M. and Edgington, B. H. .. *J. Agr. Research*, 1933, **46**, 1023.
5. Mitchell, H. H. .. Quoted from McClure, 1933.
6. Smith, M. C. and Lantz, E. M. .. *J. Biol. Chem.*, 1933, **101**, 677.
7. Hauck, H. M., Steenbock, H. and Parsons, H. T. *Am. J. Phisiol.*, 1933, **103**, 480.
8. Schulz, J. A. .. *Iowa Agr. Expt. Sta. Research Bull.*, 1938, No. 247.
9. Circla, P. .. *Chimie & Industrie*, 1938, 38, 74.
10. Wolff, A. and Kerr, E. G. .. *Am. J. Med. Sci.*, 1938, **195**, 493.
11. Muuzmdar, B. N., Ray, S. N. and Sen, K. C. .. *Ind. J. Vet. Sci. and Animal Husbandry*, 1943, **13**, 95.
12. Hammett, F. S. .. *J. Biol. Chem.*, 1925, **64**, 409, 685, 693.
13. Wadhwani, T. K. .. *Indian J. of Physiol. and Allied Sci.*, 1952, **7**, 23, 41.
14. Havesy, G., Levi, H. B. and Rebbe, O. H. *Biochem. J.*, 1940, **34**, 533.
15. Subrahmanyan, V., Duckworth, J. and Godden, W. *Ibid.*, 1939, **33**, 1421.
16. Van Slyke, D. D. and Folch, J. .. *J. Biol. Chem.*, 1940, **136**, 509.
17. Fiske, C. H. and Subbarow, Y. .. *Ibid.*, 1925, **130**, 269.
18. Darnell, M. C. (Jr.) and Walker, B. S. *Ind. Eng. Chem.*, Anal. Ed., 1940, **12**, 242.
19. Looney and Dyer .. *Jour. Lab. & Clin. Med.*, 1942, **28**, 354 (Quoted from Kolmer, J. A.. and Boerner, F., *Approved Laboratory Technique*, D. Appletion-Century Company).

AVAILABILITY OF CALCIUM IN SOME OF THE LEAFY VEGETABLES

By S. C. Devadatta, F.A.Sc. and T. C. Appanna, M.Sc.
(*Christian Medical College, Vellore*)

Received June 26, 1953

In average diets a deficiency of calcium is more likely to occur than a deficiency of any other element. The usual diet of average people in India consists mainly of cereals supplemented with pulses and vegetables. Cereals and pulses although rich in phosphorus, are poor sources of calcium. A known adequate source of calcium would be the vegetables which supplement the cereals and the pulses in the dietary. Because of the richness of calcium in milk, nutritionists have classified this food as nearly indispensable in diet. But even small amounts of milk is beyond the means of the average Indian. The problem of finding some vegetables rich in calcium which would be well utilized in the system is of paramount importance. The present investigation deals with the availability of calcium of some vegetables rich in this element which grow in abundance. Numerous studies have shown that mere measurement of the calcium content of a food is no indication of its value as a dietary source of this element. The true value depends on whether the body can utilize the calcium present in it. The classic example of this is the well-known distinction between the high calcium content of spinach and its complete unavailability to the body because of the presence of oxalic acid as demonstrated by Fincke and Sherman (1935). Rose (1920) studied the utilization of calcium of carrots with healthy young women and concluded that the calcium of carrots was nearly as well utilized as that of milk. Rose and McLeod (1923) made similar study of the utilization of calcium of almonds. When almonds furnished about 73% of the total food calcium, the calcium of the almond was almost as well utilized as that of milk, but when the almonds was made to furnish about 85% of the total food calcium, the efficiency of utilization of calcium of almond was somewhat less.

Fincke and Sherman (*loc. cit.*) made experiments with animals so that the retention of calcium could be determined by the direct analysis of the body at the end of the experiment. Rats were chosen on account of their omnivorous habit and the chemistry of their nutrition is in most respects so closely like those of human beings. The experimental animals were fed on a carefully planned diet through a period during which normal growth

and development involves a relatively large increase in the calcium content of the body. It was found by them that calcium of milk was well utilized, that of kale almost as well, while that of spinach was poorly utilized. Similar investigations were carried by Kao, Conner and Sherman (1938) and M. Speirs (1939).

Experimental

In our experiments the procedure adopted was essentially that developed by Fincke and Sherman (*loc. cit.*) in their work on the availability of calcium in spinach and kale. It was planned to determine the utilization of calcium of five Indian leafy vegetables commonly consumed by the people, *viz.*, Avati Keerai (*Sesbania grandiflora*), Mola Keerai (*Amaranthus gangeticus*), Chiru Keerai (*Amaranthus spinosus*), Karuvepilai (*Murraya kœnigii*) and Murunga Keerai (*Moringa oleifera*). The vegetables were purchased from the local market, thoroughly washed with distilled water and dried in an oven at a temperature of about 75° C. to avoid charring. The dried vegetables were thoroughly ground, mixed and bottled and stored in refrigerator. Milk was used in the form of skimmed milk powder and the same lot was used throughout the investigation. Butterfat in the form of butter purchased from local market was separately added.

The powdered dry leafy vegetables and the skimmed milk powder were analysed. The results are shown in Table I.

Table I

Analysis of Skimmed Milk and Leafy Vegetables (Dry Powder)

Composition	Skimmed Milk %	Avati Keerai %	Mola Keerai %	Chiru Keerai %	Karuvepilai %	Murunga Keerai %
Calcium ..	1·460	3·99	3·18	3·56	2·40	1·56
Phosphorus ..	0·974	0·308	0·69	0·59	1·15	0·31
Protein (N×6·25)..	39·85	29·12	33·46	31·89	17·56	25·47
Oxalic Acid ..	..	0·021	0·047	0·053	0·058	0·047

In all cases calcium was determined by the McCrudden method (1911–12) by precipitating calcium as oxalate at pH 4·8 to 5·2 using methyl red as indicator and titrating the oxalic acid with potassium permanganate solution. Phosphorus was determined by the Brigg's (1922) method. Oxalic

acid was determined by precipitation with calcium chloride in presence of acetic acid and subsequent titration with potassium permanganate solution as described by Mazumdar and De (1938).

Healthy young albino rats 28 days old reared on stock diet consisting of $\frac{2}{3}$ whole wheat, $\frac{1}{3}$ whole milk powder, vegetables with bi-weekly addition of vitamins A and D, which is known to support normal growth and calcification, were used as experimental animals. The experimental animals were put in individual iron cages.

The experimental diets were so prepared and planned that they contained almost the same percentage of calcium and phosphorus and protein. The calcium phosphorus ratio was nearly always 1 : 1. In the control diet practically all the calcium was supplied by the skimmed milk powder. In the diets containing greens, half the skimmed milk powder was replaced by enough of the green to supply an equal amount of calcium, the difference in weight being made up with maize starch.

TABLE II

Composition of Diet and their Ca and P Contents

	Control Skimmed *Milk Diet*	Avati Keerai *Diet* 1	Mola Kerai *Diet* 2	Chiru Keerai *Diet* 3	Karuvepilai *Diet* 4	Murunga Keerai *Diet* 5
Whole Wheat ..	65	65	65	65	65	65
Butterfat ..	10	10	10	10	10	10
Skimmed Milk Powder ..	22	11	11	11	11	11
Greens Powder ..	..	4·0	5·0	4·5	6·5	11·0
Salt Mixture ..	5	5	5	5	5	5
Maize Starch ..	3	9	8	8	7	3
Calcium % ..	0·341	0·327	0·331	0·338	0·345	0·319
Phosphorus % ..	0·420	0·330	0·351	0·314	0·309	0·381

The animals were so chosen that the initial weights of the groups of rats to be compared were approximately the same. Food and water was supplied *ad libitum* and records were kept for the food consumption. The experimental animals were fed the experimental diets from 28 to 60 days of age, a period during which the rate of growth and calcification is very rapid.

TABLE III

Average Growth of Rats from 28 days to 60 days on Different Diets

Diets	No. giving Average rats	Average initial weight of body	Average final body weight net	Average net gain	Average food intake	Average gain per gm. of food
		gm.	gm.	gm.	gm.	gm.
Control	4	41·5	112	72	195	0·36
1	6	38	97	59	208	0·28
2	5	34	94	60	190	0·31
3	5	37	93	56	192	0·29
4	6	33	74	41	204	0·20
5	5	38	84	46	194	0·23

At the end of the period the rats were weighed and killed with chloroform. The gastro-intestinal tract was dissected and the contents removed. The weight of the contents subtracted from the final live weight, gave the net weight which was used for calculation of the percentage of body calcium of 60 days old rats. The alimentary tract was discarded as the calcium content in the walls of intestinal tract was negligible. The rats were ashed in silica basins and finally ashed at dull red heat. The ash was dissolved in 1:4 hydrochloric acid, filtered and diluted to 500 c.c. Aliquots of this solution were analysed for calcium by the method already stated.

The results are summarized in Tables IV and V. The bodies of the albino rats on the control diet, diet 1, diet 2, diet 3, diet 4 and diet 5 contained respectively 0·764, 0·820, 0·787, 0·807, 0·906 and 0·810 per cent. of calcium. It will be noticed that the rats on the milk diet contained the lowest and those on diet 4 the highest. This is to be expected since the rats on milk diet grew faster than those on the diet 4. The intermediate values for the percentage of body calcium of rats on diet 1, diet 2, diet 3 and diet 5 were also conforming with their growth rates.

To obtain the storage of calcium it was necessary to know the initial content of calcium of the body of the animal just before the beginning of

TABLE IV

Calcium Content of 60 days old Rats fed on Diets containing the same amount of Calcium from Milk or Vegetables

Diets	No. giving Average rats	Average net body weight (gm.)	Average Calcium intake (gm.)	Average total body calcium (gm.)	Average % body calcium
Control	4	112	0·625	0·856	0·764
1	6	97	0·690	0·798	0·820
2	5	94	0·650	0·741	0·787
3	5	93	0·654	0·751	0·807
4	6	74	0·710	0·671	0·906
5	5	84	0·620	0·681	0·810

TABLE V

Calcium Content of 60 days old Rats on Various Diets and thier Utilization Factor

Diets	No. giving average rats	Calcium in body at 60 days		Calcium at 28 days (gm.)	Calcium retained in body (gm.)	Calcium in food eaten (gm.)	Utilization factor
		Total (gm.)	% net wt.				
Control	4	0·856	0·764	0·223	0·533	0·625	0·85
1	6	0·798	0·820	0·283	0·515	0·690	0·74
2	5	0·741	0·787	0·226	0·515	0·650	0·78
3	5	0·751	0·807	0·200	0·521	0·654	0·79
4	6	0·671	0·906	0·286	0·305	0·710	0·54
5	5	0·681	0·810	0·280	0·401	0·620	0·69

the experiment. This was obtained by determining the per cent. calcium of the body at 28 days of age.

For an exact basis of comparison a " calcium utilization factor " was calculated for each of the diets. This was obtained by dividing the calcium retained by the calcium intake. The results are given in Table V. The difference of the utilization factor of calcium of diet 4 from that of milk is 0·310. This value is quite significant and therefore the calcium of diet 4 is not quite as well utilized as that of milk. For diet 5 the difference for

utilization factor is 0·160 which is not very far from the figure 0·11 for diet 1. Nevertheless these two vegetables, Karuvepilai and Murunga Keerai serve as a fairly good source of calcium. On the diet 1 the difference of calcium utilization factor from that of milk is 0·11. This figure is not very significant and it appears that the calcium of diet 1 is almost as well utilized as that of milk.

The two species of Amaranthus, Mola Keerai and Chiru Keerai, included in the diets 2 and 3 respectively contain a high amount of calcium, 0·54% and 0·47% respectively. In spite of the fairly high percentage of oxalic acid, these are well utilized and the utilization factor being 0·78 and 0·79 respectively. With a difference of 0·07 and 0·06 from that of the utilization factor of milk at 0·85, they are as well utilized as that of milk.

Summary

Healthy young rats, 28 days old, were placed on six diets in one of which all the calcium was supplied entirely by skimmed milk. In the other diets half of the skimmed milk was replaced by enough ground dried leafy vegetables to provide the same amount of calcium as in the milk diet. At 60 days of age the animals were killed and their bodies analysed for calcium. Comparison of the availability of calcium in these vegetables with that of milk was made by calculating for each an utilization factor which is the ratio of calcium retention to intake. The values for the six diets were: 0·85 for milk diet, 0·74 for diet 1, 0·78 for diet 2, 0·79 for diet 3, 0·54 for diet 4, and 0.69 for diet 5 respectively.

All the five leafy vegetables, *viz.*, Avati Keerai (*Sesbania grandiflora*), Mola Keerai (*Amaranthus gangeticus*), Chiru Keerai (*Amaranthus spinosus*), Curry leaves (*Murraya Kœnigii*), Murunga Keerai (*Moringa oleifera*) form good sources of calcium from the point of nutrition, especially Avati Keerai, Mola Keerai and Chiru Keerai, as the calcium in these are used as well as that in milk.

Names of the Vegetables and Equivalents in other Important Indian Languages

	Botanical Name	Tamil	Kannada	Hindustani
1.	*Sesbania grandiflora* ..	Avati Keerai	Agase	Agasthi
2.	*Amaranthus gangeticus* ..	Mola Keerai	Yeledantu	Lal Choalai
3.	*Amaranthus spinosus* ..	Chiru Keerai	..	..
4.	*Murraya kœnigii* ..	Karuvepilai	Karibevu	Gandhela
5.	*Moringa oleifera* ..	Murunga Keerai	Murige	Saijan

REFERENCES

1. Brigg .. *J. Biol. Chem.*, 1922, **53**, 13.
2. Fincke and Sherman .. *Ibid.*, 1935, **110**, 421.
3. Kao, Conner and Sherman .. *Ibid.*, 1938, **123**, 221.
4. McCrudden .. *Ibid.*, 1911–12, **10**, 187.
5. Mazumdar and De .. *Ind. Jour. Med. Res.*, 1938, **25**, 271.
6. Rose .. *J. Biol. Chem.*, 1920, **41**, 349.
7. ——— and McCleod .. *Ibid.*, 1923, **57**, 277.
8. M. Speirs .. *J. Nutr.*, 1939, **17**, 557.

ROOTING RESPONSES IN ISOLATED TROPICAL LEAVES

By B. Samantarai and T. Kabi
(*Department of Botany, Ravenshaw College, Cuttack*)

Received September 1, 1953
(Communicated by Dr. I. Banerji, F.A.SC.)

Introduction

The scope of using isolated leaves in the study of plant physiology has been suggested by Gregory and Samantarai (1950). While inducing a bunch of roots in the detached leaves of dwarf french bean and ivy and studying the various biochemical aspects they come to the important conclusion that these living leaves are miniature plants in themselves and as such a better insight into the metabolic conditions of a living cell can be obtained in them since the complexities introduced by processes of translocation and storage are greatly minimised. The present investigation was undertaken to study how far such induction of roots is possible in the tropical leaves. Samantarai, Misra and Kabi (1953) and Samantarai and Misra (1953) have published brief reports on the rooting responses of a few tropical leaves.

Material and Method

The isolated leaves of the following plants were selected for the purpose. *Ipomœa batatas, Helianthus annuus, Chenopodium album, Amaranthus gangeticus, Cephalandra indica, Boerhaavia diffusa* and *Pogostemon plectranthoides.*

Three synthetic hormones, *viz.*, β-indolyl acetic acid (I.A.A.), β-indolyl butyric acid (I.B.A.) and α-Naphthylacetic acid (N.A.A.) were used for the leaves of *Ipomœa batatas*, and for the rest of the leaves only I.B.A. was used. The aqueous solution of the hormone was prepared according to the suggestions of Gregory and Samantarai (1950). Solutions of different concentrations as 100, 50, 20, 10, 5 and 2·5 p.p.m. (parts per million) were made by diluting the stock solution. Ten leaves of approximately same age and size were selected and collected from each plant and treated with the different concentrations of the hormone. One set of each was kept in distilled water as control. After 24 hours the leaves were washed thoroughly and kept in a humid propagating chamber with their petioles dipped in tap water upto a height of 3 cms. Water was changed twice daily in order to prevent

the growth of micro-organisms. For each treatment 10 replicates were used. The final rooting response was measured by the percentage of leaves rooted, the average days from treatment to first root emergence, average number of roots per leaf and the fresh weight of the roots produced. Where the leaves were intended for planting in soil the total length of the roots produced per leaf was recorded.

EXPERIMENTAL RESULTS

After certain preliminary experiments it was found that *Ipomœa* leaves, from the fifth node from apex, is quite suitable as it is not tender to the toxic effect of the hormone and is of vigorous growth. These leaves responded best for rooting. With such leaves various concentrations of the three hormones were tried to determine the optimal concentrations for root production.

TABLE

Effect of Different Hormones at Different *ntrations on Root Production in* Ipom

Points of observation	Hormone applied	Concentration of the Hormone, in p.p.m.						
		100	50	20	10	5	2·5	0 (control)
Percentage of leaves rooted	I.A.A.	100	60	30	30	40	30	10
	I.B.A.	40	70	90	100	100	100	0
	N.A.A.	90	90	60	50	50	50	20
Average days from treatment to root-emergence	I.A.A.	12	12	15	15	15	15	22
	I.B.A.	12	12	9	8	8	6	—
	N.A.A.	9	9	11	12	12	14	25
Average number of roots per leaf	I.A.A.	6	4	3	3	3	3·5	0·5
	I.B.A.	2·5	6	7·5	8	8	8	0
	N.A.A.	4	5	3	2	2	2	1
Average weight of roots in mgm. per 10 leaves	I.A.A.	930	580	210	138	140	112	15
	I.B.A	225	820	925	1030	1010	1350	0
	N.A.A.	540	630	290	65	70	80	20

The effects of the three hormones can be seen from Table I. The optimal concentration of I.A.A., I.B.A. and N.A.A. are 100 p.p.m., 2·5 p.p.m. and 50 p.p.m. respectively. The best rooting material in this case is evidently

TABLE II

Effect of Various Concentrations of I.B.A. on the other Six Species of Leaves

Points of observation	Name of the leaves	Concentration of the Hormone in p.p.m.						
		100	50	20	10	5	2·5	0 (control)
Percentage of leaves rooted	*Helianthus*	40	70	90	100	100	90	30
	Pogostemon	30	50	80	90	100	100	30
	Amaranthus	0	40	80	100	100	90	30
	Chenopodium	50	70	90	100	100	90	20
	Boerhaavia	80	90	100	100	100	100	40
	Cephalandra	90	100	100	100	90	80	20
Average days from treatment to root emergence	*Helianthus*	12	12	10	10	10	10	24
	Pogostemon	8	8	8	6	5	5	20
	Amaranthus	—	8	8	7	7	7	18
	Chenopodium	14	14	14	11	11	12	23
	Boerhaavia	9	11	11	9	12	12	20
	Cephalandra	14	14	12	13	13	13	23
Average number of roots per leaf	*Helianthus*	15	30	40	67	65	52	10
	Pogostemon	35	40	70	72	80	85	15
	Amaranthus	0	8	10	15	17	12	3
	Chenopodium	6	9	10	16	12	11	2
	Boerhaavia	2	3	4	5	4·5	5	0·5
	Cephalandra	3·5	4	6	6	5	5	0·2
Average length of roots in cm. per leaf	*Helianthus*	32	41·1	72·4	122	110·5	102	22
	Pogostemon	69·5	78·2	114	135	152·5	165	36
	Amaranthus	0	3·5	6·2	18	22·4	20·1	3·6
	Chenopodium	19	24·3	31	50·5	40·2	36	6
	Boerhaavia	3	3·5	6	8·8	8	6·7	2
	Cephalandra	6	9·2	13	12	9·4	9·8	2·1

I.B.A. The other effects of the three hormones produced on these isolated leaves are of much interest.

Leaves treated with 100 and 50 p.p.m. of I.A.A. showed swellings in the petioles one week after the treatment. After 12 days roots emerged from the swollen portions of those leaves and the leaves treated with other concentrations rooted after 15 days. In the control leaves roots emerged only in a few cases after 22 days. Leaves treated with I.B.A. showed swellings in almost all the leaves except the untreated leaves after 4 days. Petioles of the leaves treated with higher concentrations, *i.e.*, 100 and 50 p.p.m. began to yellow and rot after one week and after the second week 60% of the leaves from 100 p.p.m. and 30% from 50 p.p.m. turned yellow and rotted away. Emergence of roots was first observed in the leaves treated with 2·5 p.p.m. after 6 days. Roots did not emerge from the controls till the 25th day. Leaves treated with 100 and 50 p.p.m. N.A.A. first showed swellings in the petioles after 5 days and the rest after 9 days. Very few of the control leaves rooted after 25 days. 90% of the leaves treated with 100 and 50 p.p.m. rooted after 9 days. None of the leaves treated with this hormone showed signs of decay.

Experimental data for the other 6 leaves *Helianthus*, *Pogostemon*, *Amaranthus*, *Chenopodium*, *Boerhaavia* and *Cephalandra* are given in Table II.

The selection of material in these plants was made with the characteristics that the leaves were not tender and at the same time at a stage of vigorous growth. It will be clear from Table II that cent per cent. of the *Helianthus* leaves rooted in 10 and 5 p.p.m. I.B.A. The first root appearance was also earlier in these concentrations than in the others. In 100 and 50 p.p.m. 60 and 30% of the leaves respectively turned yellow and rotted away. The optimum concentration is 10 p.p.m.

The leaves of *Pogostemon* responded better to lower concentrations. In higher concentrations as 100 and 50 p.p.m. 70 and 50% of the leaves respectively turned yellow and rotted away. Here the optimum concentration is 2·5 p.p.m.

All the leaves of *Amaranthus* treated with 100 p.p.m. and 60% of the leaves treated with 50 p.p.m. rotted away. The leaves treated with 5 p.p.m. rooted earlier and the average number and length of roots prove this concentration to be the optimum.

The leaves of *Chenopodium* showed best rooting in 10 p.p.m. 50% of the leaves treated with 100 p.p.m. rotted away.

100% of the leaves of *Boerhaavia* rooted in all concentrations from 2·5 to 20 p.p.m. but first root emergence was observed in 10 p.p.m. In the controls 40% of the leaves rooted but roots appeared very late. 10 p.p.m.

is the optimum concentration. No toxic effect of higher concentrations of the hormone was seen here.

All the leaves of *Cephalandra* rooted in 10, 20 and 50 p.p.m. but roots appeared earlier in 20 p.p.m. which is the optimum concentration. Even in the strongest concentration the leaves did not show any signs of deterioration.

Discussion

The leaves which were studied for rooting response are mostly herbaceous of which *Helianthus*, *Amaranthus*, *Chenopodium* and *Ipomœa* are annuals and the rest are perennials. Even in those herbaceous perennials *Boerhaavia* is pretty tough and *Cephalandra* which is a perennial climber is also very tough. The delicate or the tough habit of the plant is to a certain extent responsible for the reactions of the hormones applied. According to Went and Thimann (1945) the hormones bring about the plasticity of the cell walls, so that meristematic cells grow out of the older cells. Gregory and Samantarai (1950) have also shown that the meristematic cells originate at the back of the vascular bundles in the petioles of dwarf french bean and ivy. These form the root primordia and later on when the root grows out the xylem and the phloem of the root establish connections with those of the petioles and this can only happen by the cell structures of the xylem and phloem elements of the petioles undergoing plasticity. If the cell walls are more of gell condition or are thick enough, which seem to be the case with *Cephalandra*, higher concentrations of auxins are necessary to bring about the initiation of the roots, whereas in the more delicate leaves like *Ipomœa*, *Pogostemon*, etc., lower concentrations are good enough for the rooting. At higher concentrations these hormones prove toxic to certain leaves. Gregory and Samantarai have shown that by the application of synthetic hormones hydrolysis of nitrogenous material and carbohydrates takes place and translocation of the hydrolytic products occurs to the petioles from the lamina. It may therefore be said that the toxic effect may be due to complete hydrolysis and translocation of foodstuff from the lamina to the petiole and the hormone acting as a poison at a higher concentration.

Another feature is that the effect of all the hormones is not the same. So far as *Ipomœa* is concerned I.B.A. seems the most reactive in producing roots. It may be due to the constitution of the hormone but at this stage statements of this nature are mere speculations. Leaves which were rooted by the application of the synthetic hormones are living and are healthy after being planted in soil. The anatomy of these rooted leaves grown in soil is engaging the attention of the authors (Samantarai and Kabi, 1953) for better understanding of the processes concerned.

SUMMARY

Isolated leaves from seven different plants were treated with hormones in order to study their rooting response.

Ipomœa batatas leaves were treated with I.A.A., I.B.A. and N.A.A. and it was found that 2·5 p.p.m. I.B.A. is the best concentration for rooting.

Pogostemon leaves showed best rooting with 2·5 p.p.m. I.B.A., *Amaranthus* with 5 p.p.m., *Helianthus*, *Chenopodium* and *Boerhaavia* with 10 p.p.m. and *Cephalandra* with 20 p.p.m. I.B.A.

Rooting response of various experimental leaves is different to different concentrations of the hormones.

It is suggested that the different rooting response of the materials to the different hormones lies on the structure of the cell wall and the reactive capacity of the hormones.

REFERENCES

Gregory, F. G. and Samantarai, B. .. "Factors concerned in the rooting responses of isolated leaves," *Journ. Expt. Bot.*, 1950, **1**, 159–93.

Samantarai, B. and Kabi, T. .. "Secondary growth in petioles and the partial shoot theory of the leaf," *Nature*, 1953, **172**, 37.

——— and Misra, G. .. "Hormone-induced rooting in isolated leaves," *Science & Culture*, 1953, **18**, 388–89.

———, Misra, G. and Kabi, T. .. "Studies in rooting response," *Curr. Sci.*, 1953, **22**, 48.

Went, F. W. and Thimann, K. V. .. *Phytohormones*, 1945, Macmillan Co., New York.

A CONTRIBUTION TO THE EMBRYOLOGY OF *OLAX WIGHTIANA* WALL.

By S. Shamanna

(*Department of Botany, Central College, Bangalore*)

Received December 9, 1953

(Communicated by Prof. L. Narayana Rao)

Introduction

The present work on the embryology of *Olax wightiana* is part of a detailed investigation which was originally undertaken by Prof. L. Narayana Rao, Head of Department of Botany, University of Mysore, Central College, Bangalore, to include the embryological studies on the several allied families, belonging to Santalales. A large collection of several representative forms was made from time to time by Prof. Rao, who subsequently transferred to the present author, some of the materials of *Olax wightiana*. To this was added by the author, more material of the plant, and Prof. Rao most generously suggested, that this work be completed by the author himself. Grateful acknowledgment is here made, for this kind gesture, extended by Prof. Rao, and for the many helpful and timely comments and criticisms that he freely offered during the progress of the present investigation.

According to Engler and Prantl (1894) the genus *Olax* is included in the *sub-family* Olaceæ of the family Olacaceæ of the order Santalales. It consists of 35 paleotropical species (Willis, 1948). Gamble (1915) records four species in South India.

The Olacaceæ is a very interesting family consisting of trees, shrubs and vines. Like other closely allied families, the members of this family are partial parasites. Previous work on the allied species *Olax imbricata* is by Fagerlind (1947). *Olax wightiana*, which is the member of the present study, is a climbing shrub.

Materials and Methods

Material was collected at Nandi hills, and was fixed in Formalin-acetic-alcohol, dehydrated and embedded in paraffin, according to the customary practice, and sectioned at a thickness of 10–20 microns. The sections were stained in Heidenhain's iron-alum hæmotoxylin with erythrosin in clove oil as the counterstain.

In order to excise the embryo sac the technique recently outlined by Paliwal (1953) was tried and temporary wholemounts of embryo sac in lactophenol were made to obtain the full view of the mature embryo sac. Pollen grains were similarly examined to observe the nature of the wall and the number of pores.

FLOWER

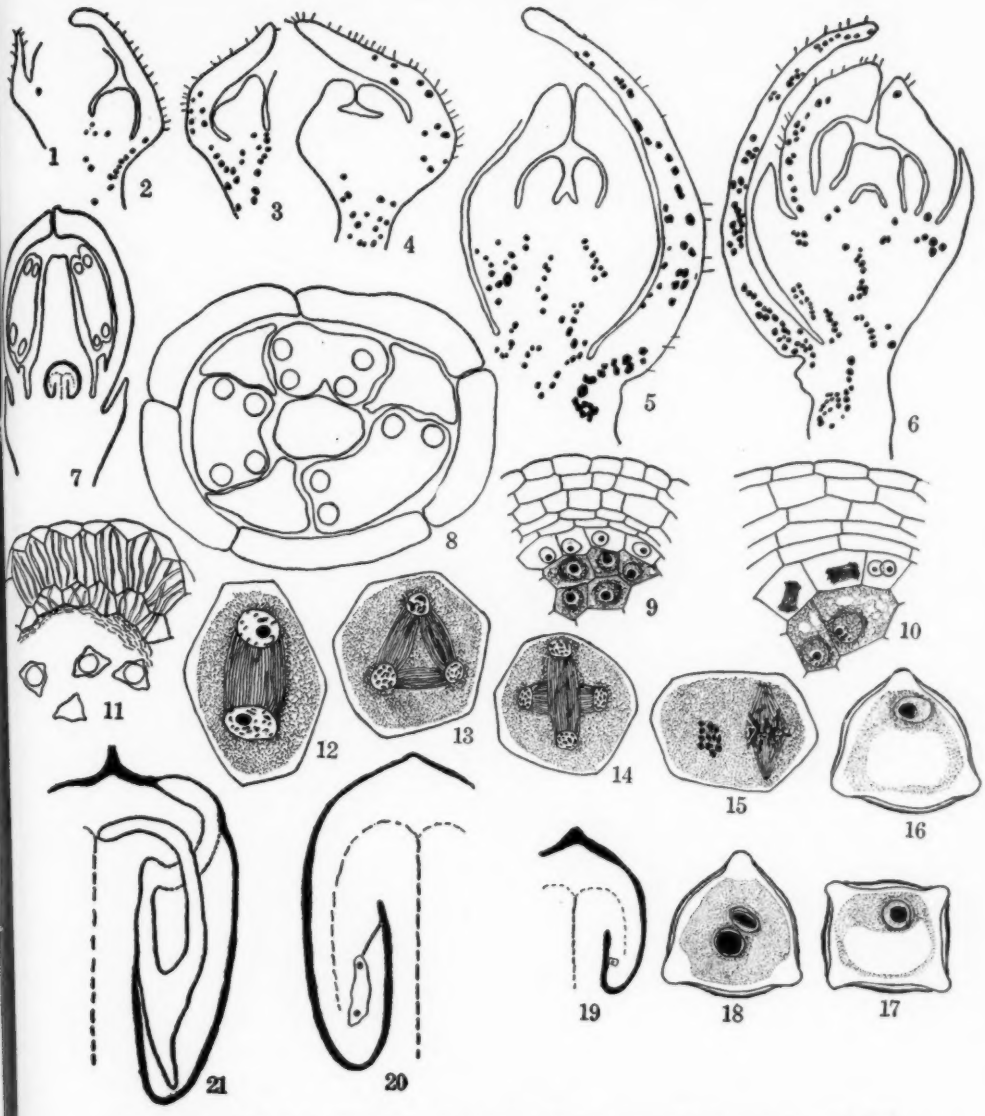
The floral parts arise in acropetal succession (Figs. 1–6). Figs. 7 and 35 represent the longitudinal section of a young flower bud showing the disposition of the different floral parts. The flowers are bisexual, in axillary racemes, and minutely bracteate. Unicellular hairs arise as prolongations of epidermal cells and are distributed over the floral parts. The sepals are fused to form a cupular calyx. In a fully grown bud, the corolla tube is narrow at the base, slightly widens above, then narrows again, and terminates in a club-shaped apex. As the flower opens, the lobes of the corolla become strongly reflexed above the swollen region. The corolla lobes are five in number, showing a valvate æstivation. Three fertile stamens alternate with three staminodes (Fig. 8) and they are epipetalous. A long style arises from the centre of the ovary and ends in a trilobed stigma. The stigmatic lobes have papillate protrusions. Cells filled with tannin are seen in profusion, distributed in the floral parts and they increase gradually during the development of the flower.

MICROSPORANGIUM

A transverse section of the young anther lobe shows a group of microspore mother cells sorrounded by tapetum, three middle layers, endothecium and epidermis. The cells of the tapetum are uninucleate at first (Fig. 9) but later become binucleate (Fig. 10) and are of the glandular type. In the mature anther, the tapetum becomes disorganised. The endothecium and the layer immediately below it develop fibrillar thickenings (Fig. 11). The microspore mother cells are polygonal with a prominent nucleus. After undergoing the usual meiotic divisions they form the dyads (Fig. 12) and then the tetrads of microspores. The microspores are arranged tetrahedrally (Fig. 13) but occasionally show a decussate arrangement (Figs. 14, 15).

MALE GAMETOPHYTE

The exine is uniformly thick except at the pores, where only the intine is present. Pollen grains with three or four pores are seen. The nucleus in the young microspore is situated in the centre. Subsequently it moves towards the wall, leaving a large vacuole in the centre (Figs. 16, 17). The division of the nucleus of the microspore results in the formation of a tube



FIGS. 1–21. Figs. 1–6. Development of floral parts in acropetal succession, ×40 each. Fig. 7. L.S. of Young flower bud, ×12. Fig. 8. T. S. of flower bud showing stamens alternating with staminodes, ×28. Fig. 9. Portion of anther lobe showing epidermis, endothecium, middle layers, uninucleate tapetum and sporogenous cells, ×315. Fig. 10. Same at later stage showing binucleate tapetum, ×315. Fig. 11. Portion of anther wall showing endo-

thecium and next middle layer fibrillar, ×240. Fig. 12. First division in microspore mother cell, ×900. Fig. 13. Tetrahedral disposition during second division of microspore mother cell, ×900. Figs. 14–15. Decussate disposition during second division of microspore mother cell, ×900. Figs. 16–17. Uninucleate pollen grains with three and four pores, ×675. Fig. 18. Mature pollen grain, ×675. Figs. 19–21. Stages in the development of ovule, ×40 each.

nucleus and a generative nucleus. The pollen grain is two-celled, with rich cytoplasm, and devoid of vacuolation at the time of dehiscence of the anther (Fig. 18).

OVARY

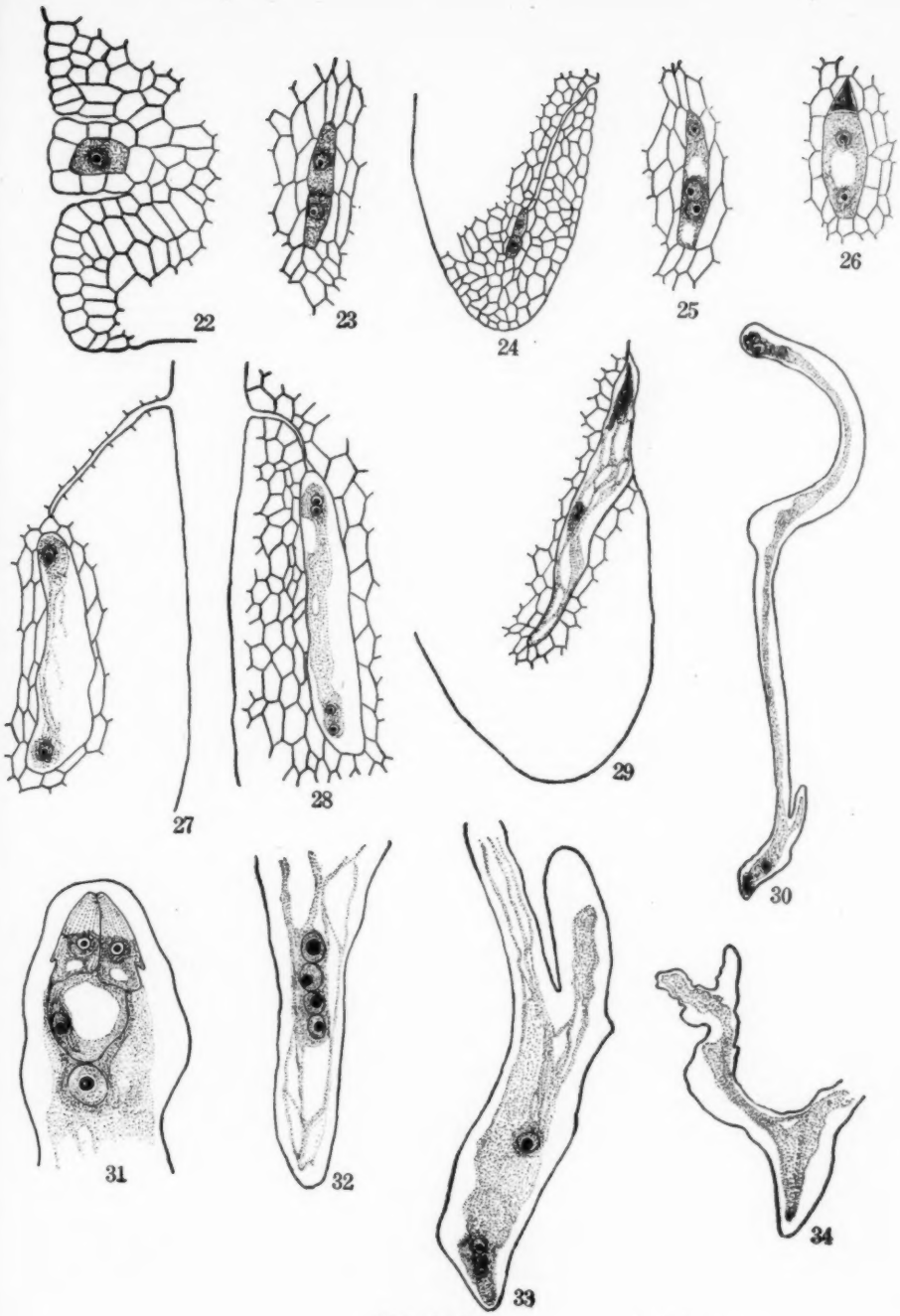
The ovary is superior, and is surrounded by a hypogynous disc. A small mound of tissue arises from the base of the ovary and forms the mamelon. As development proceeds, the mamelon elongates in length to form the central column. Three pendulous ovules arise from the mamelon, and ultimately project into the ovarian cavity around the central column. As regards the nature and morphology of the mamelon and the ovules in the several related families of the Santalales, reference may be made here to the recent paper by Maheshwari and Singh (1952). The ovules in the case of *Olax wightiana* are clearly unitegmic, although this condition may not appear to be distinct during its subsequent growth in the ovarian cavity. The wall of the ovary and the central column are closely apposed to the ovules, which are at first hemianatropous. The pronounced growth of the funiculus brings about a change in the orientation of the ovules, which subsequently take a bend in the available space around the central column, where there appears to be the least resistance for the growth of the ovules causing them to become anatropous (Figs. 19–21). A single conducting strand runs along the central column, and branches into each of the pendulous ovules.

MEGASPOROGENESIS

A single archesporial initial differentiates in the hypodermal layer, and is distinguishable from the neighbouring cells by the large size, dense cytoplasm, and a prominent nucleus (Figs. 22 and 36). The archesporial cell functions directly as the megaspore mother cell. It divides in turn to form the dyads (Figs. 23 and 38). By now the ovules assume the anatropous condition, and a narrow micropylar canal is formed (Figs. 24, 37 and 39). The upper dyad without dividing further begins to degenerate, while the nucleus of the lower dyad undergoes three successive divisions to produce an eight-nucleate embryo sac (Figs. 25–30) of the Allium type (Maheshwari, 1948).

EMBRYO SAC

A two-nucleate embryo sac, with the upper dyad degenerating is shown in Figs. 25, 26, 40 and 41. The embryo sac begins to enlarge, and elongates



FIGS. 22–34

FIGS. 22–34. Fig. 22. Ovule showing an archesporial cell, ×315. Fig. 23. Formation of dyad, ×315. Fig. 24. Dyad in relation to anatropous ovule and micropylar canal, ×135. Fig. 25. Early two-nucleate embryo-sac with undivided upper dyad, ×315. Fig. 26. Slightly later stage showing degenerated upper dyad and two-nucleate embryo sac with a central vacuole, ×315. Fig. 27. Late two-nucleate embryo sac, ×225. Fig. 28. Four-nucleate intraovular embryo sac, ×225. Fig. 29. Embryo sac, becoming extra ovular, ×225. Fig. 30. Embryo sac with its full complements, ×80. Fig. 31. Egg apparatus enlarged showing filiform apparatus in synergids, ×339. Fig. 32. Lower quartet of embryo sac enlarged, ×315. Fig. 33. An enlarged sketch of the lower end of embryo sac shown in Fig. 30, ×225. Fig. 34. Branched and lobed nature of cæcum, ×100.

considerably during its growth in subsequent stages. The micropylar and the chalazal nuclei in turn divide to produce the four-nucleate embryo sac. So far the embryo sac appears a simple tubular structure, and remains intra ovular (Figs. 27, 28 and 42). Soon the two ends of the embryo sac begin to elongate, the micropylar end elongating more than the chalazal end, and becoming extra ovular (Figs. 29 and 43). The micropylar end of the embryo sac, emerging out of the ovule, begins to grow upwards between the inside of the ovule and the central column. Then it takes a bend almost perpendicular to the latter and proceeds forwards. Again it takes a bend upwards, and running along the wall of the ovary, reaches the base of the style. It may even travel upwards a little into the style. It is at this end of the embryo sac that the egg apparatus is organised. The synergids are hooked, and exhibit a filiform apparatus and the egg cell lies close to them, partly covered above by their basal portions (Fig. 31). The upper polar nucleus is at first close to the egg, but later migrates downwards. The chalazal quartet is formed at the lower end of the embryo sac, inside the ovule (Fig. 32). Slightly above the level of the antipodal nuclei, the embryo sac now produces a short outgrowth (Fig. 33), which begins to grow hereafter prominently as a cæcum. This cæcum continues to grow in an upward direction in the ovule, until it reaches the base of the ovule, where it takes a sharp turn at right angles, and growing beyond in a lateral direction meets the vascular strand at the apex of the mamelon (Fig. 21). The cæcum evidently has a prominent haustorial function which is further evidenced by its lobed and branched nature as well (Fig. 34). In this connection it may be noted, that in the Santalaceæ, the chalazal end of the embryo sac grows into the tissue of the ovule and finally reaches the placental column, while the micropylar end becomes extra ovular, and grows into the ovarian cavity (Iyengar, 1937; Rao, 1942).

DISCUSSION

The hypodermal archesporial cell functions directly as the megaspore mother cell as in *Olax imbricata*. The development of the embryo sac in

Olax imbricata conforms to the Polygonum type (Fagerlind, 1947). It is therefore interesting to note that in the related species now investigated, *viz.*, *Olax wightiana* the embryo sac is formed according to the Allium type. Maheshwari (1948) states " .....the minor floral differences by which many species are distinguished are not reflected in the embryo sac. For this reason, embryo sacs of species within the same genus usually do not differ markedly from one another and frequently (but not always) the genera belonging to a small family or tribe also show a close similarity. When this is not the case the position needs careful scrutiny, which may lead to a reorientation of our ideas of the relationships or may suggest new groupings". The present observations therefore raise points for discussion on the relationships of the different types of embryo sacs. A study of the other uninvestigated species belonging to this genus may throw further light on the question of the origin and development of the embryo sac and the relationships within this genus.

Summary

The floral parts arise in acropetal succession. Unicellular hairs are distributed over the floral parts. Plenty of tannin cells are found.

The wall of the anther consists of five layers external to the tapetum. The tapetal cells are uninucleate at first, but later become binucleate, and are of the glandular type. The endothecium and the layer immediately below it are fibrillar. The microspores are arranged tetrahedrally, but occasionally show a decussate arrangement. The pollen grains have three and occasionally four pores and they are two-celled at the time of shedding.

The superior ovary is surrounded by a hypogynous disc. Three pendulous ovules arise from the mamelon, and project into the ovarian cavity around the central column.

The ovules are unitegmic, although this condition may not appear to be distinct during their subsequent growth, and they are tenuinucellate. At first they are hemianatropous, but later become anatropous.

A single archesporial cell functions directly as the megaspore mother cell. The development of the embryo sac conforms to the Allium type, and has been studied in detail. The embryo sac develops a cæcum within the ovule, which assumes a prominent haustorial function.

Acknowledgment

In conclusion, it is my pleasent duty to record my gratitude to Prof. L. Narayana Rao and Dr. S. B. Kausik, for their kindness, constant encouragement, and valuable guidance. I am indebted to the authorities of the

University of Mysore, for the award of the Senior Research Training Scholarship of Government of India, which provided me the opportunity and facilities to work. I am also thankful to Mr. M. M. Veerabhadriah for helping me in taking photomicrographs.

LITERATURE CITED

Engler, A. and Prantl, K. .. *Die Natürlichen Pflanzenfamilien*, 1894, **3**, 231–42.

Fagerlind, F. .. "Gynöeceummorphologische und embryologische studien in der Familie Olacaceæ," *Bot. Notiser*, 1947, 207–30.

Gamble, J, S. .. *Flora of the Presidency of Madras*, 1915, **1**, London.

Iyengar, G. S. .. "Life-history of *Santalum album* Linn.," *J. Ind. Bot. Soc.*, 1937, **16**, (4), 175–95.

Maheshwari, P. .. "The Angiosperm embryo sac," *Bot. Rev.*, 1948, **14**, 1–56.

——— and Singh, B. .. "Embryology of *Macrosolen cochinchinensis*," *Bot. Gaz.*, 1952, **114** (1), 20–32.

Paliwal, R. L. .. "A new technique for the micro-dissection of embryo sacs of the Santalaceæ," *Phytomorphology*, 1953, **3** (1, 2), 118–21.

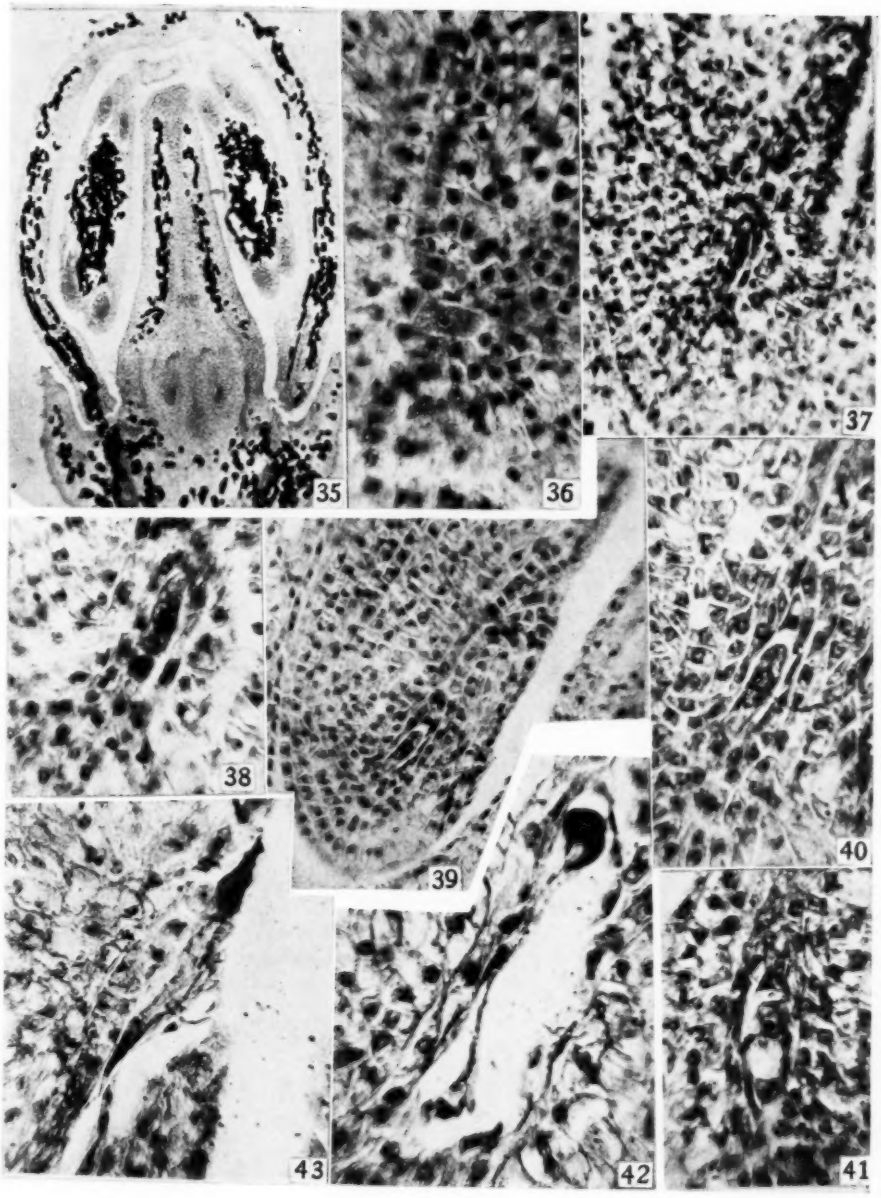
Rao, L. N. .. "Studies in Santalaceæ," *Ann. Bot.*, 1942, **6**, 151–75.

Willis, J. C. .. *A Dictionary of the Flowering Plants and Ferns*, Cambridge Uni. Press, London, 1951.

EXPLANATION OF PLATE

FIGS. 35–43. Fig. 35. L.S. of flower bud, ×31. Fig. 36. L.S. of ovule showing an archesporial cell, ×400. Fig. 37. L.S. of ovule showing dyads, ×220. Fig. 38. Dyads in Fig. 37 enlarged, ×400. Fig. 39. L.S. of ovule showing degenerating upper dyad and nucleus of lower dyad divided, ×220. Fig. 40. Dyads of Fig. 39 enlarged showing nucellar epidermis, degenerating upper dyad and two-nucleate lower dyad, ×400. Fig. 41. Upper dyad degenerated forming a cap and lower two-nucleate embryo sac with a central vacuole, ×400. Fig. 42. Two-nucleate, embryo sac elongating and intra-ovular, ×400. Fig. 43. Four-nucleate embryo sac becoming extra-ovular, ×400.

906–54 Printed at the Bangalore Press, Bangalore City, by C. Vasudeva Rao, Superintendent and Published by The Indian Academy of Sciences, Bangalore


35
36
37
38
39
40
41
42
43

INDEX TO VOL. XXXIX (B)

AUTHOR INDEX

Alikunhi, K. H. and Chaudhuri, H. — On the life-history and bionomics of the Carp Minnow, *Chela phulo* (Hamilton), 76.

Appanna, T. C. .. *See* Devadatta and Appanna.

Banerji, I. and Hakim, A. — A contribution to the life-history of *Artocarpus lakoocha* Roxb., 128.

Bhatnagar, V. B. .. *See* Choudhri and Bhatnagar.

Chacko, P. I. and Srinivasan, R. — Fish mortality in the rock pools of a quarry near Virudhunagar (Ramanathapuram District), Madras, 91.

Chaudhuri, H. .. *See* Alikunhi and Chaudhuri.

Choudhri, R. S. and Bhatnagar, V. B. — Effect of maleic hydrazide on growth, bolting behaviour and keeping quality of Indian radish (*Raphanus sativus* L.), 1.

Date, W. B. and Hansen, Elmer — Pectic changes in pears during storage and ripening, 171.

Devadatta, S. C. and Appanna, T. C. — Availability of calcium in some of the leafy vegetables, 236.

Ganguly, D. .. *See* Padmanabhan and Ganguly.

Hakim, A. .. *See* Banerji and Hakim.

Hansen, Elmer .. *See* Date and Hansen.

Jones, S. .. *See* Pantulu and Jones.

Jotwani, M. G. .. *See* Pradhan and Jotwani.

Kabi, T. .. *See* Samantarai and Kabi.

Mahabale, T. S. and Solanky, I. N. — Studies in the chenopodiaceæ, II (212).

Mathew, K. T. .. Studies on the black rot of coffee, I, II, 133, 179.

Mirashi, M. V. .. Contribution to our knowledge of the physiological anatomy of some Indian hydrophytes, I, 114.

Misra, J. N. and Ranganathan, V. — Digestion of cellulose by the mound building termite, *Termes* (*cyclotermes*) *obesus* (Rambur), 100.

Padmanabhan, S. Y. and ganguly, D.		Relation between the age of rice plant and its susceptibility to helminthosporium and blast diseases, 44.
Pantulu, V. R. and Jones, S.		On some metamorphosing stages of eels (Muræenidæ) from the estuary of the Burhabulong river, Orissa State, 24.
Pradhan, S. and Jotwani, M. G.		Studies on some aspects of bioassay of stomach poisons, 37.
Ranganathan, V.	..	*See* Misra and Ranganathan.
Rao, C. Venkata	..	A contribution to the embryology of Bombacaceæ, 51.
Samantarai, B. and Kabi, T.		Rooting responses in isolated tropical leaves, 243.
Shamanna, S.	..	A contribution to the embryology of *olax wightiana* Wall., 249.
Solanky, I. N.	..	*See* Mahabale and Solanky.
Sreenivasan, A.	..	*See* Venkataraman and Sreenivasan.
Srinivasan, R.	..	*See* Chacko and Srinivasan.
Subrahmanyan, R.	..	A new member of the Euglenineæ, *Protoeuglena noctilucæ* gen. et. sp. nov., occurring in *Noctiluca miliaris* Suriray, causing green discoloration of the sea off Calicut, 118.
Venkataraman, R. and Sreenivasan, A.		Studies on the red halophilic bacteria from salted fish and salt, 17.
Wadhwani, T. K.	..	Effect of fluorine on the composition of bones: changes in the composition of bones of rats, I, 223.

TITLE INDEX

Artocarpus lakoocha Roxb., life-history, a contribution (Banerji and Hakim), 128.

Black rot of coffee, studies, I, II (Mathew), 133,179.

Bombacaceæ, embryology, a contribution (Rao), 51.

Bones, composition, effect of fluorine: changes in the composition of bones of rats, I (Wadhwani), 223.

Calcium in some of the leafy vegetables, availability (Devadatta and Appanna), 236.

Carp Minnow, *chela phulo* (Hamilton), on the life-history and bionomics (Alikunhi and Chaudhuri), 76.

Chenopodiaceæ, studies, II (Mahabale and Solanky), 212.

Eels (Murænidæ) from the estuary of the Burhabulong river, Orissa State, some metamorphosing stages (Pantulu and Jones), 24.

Fish mortality in the rock pools of a quarry near Virudhunagar (Ramanathapuram District), Madras (Chacko and Srinivasan), 91.

Hydrophytes, some Indian, physiological anatomy, contribution to our knowledge, I (Mirashi), 114.

Olax wightiana Wall., embryology, a contribution (Shamanna), 249.

Pears, pectic changes, during storage and ripening (Date and Hansen), 171.

Protoeuglena noctilucæ gen. et sp. nov., a new member of the Euglenineæ, occurring in *Noctiluca miliaris* Suriray, causing green discoloration of the sea off Calicut (Subrahmanyan), 118.

Radish, Indian (*Raphanus sativus* L.), growth, bolting behaviour and keeping quality, effect of maleic hydrazide (Choudhri and Bhatnagar), 1.

Red halophilic bacteria from salted fish and salt, studies (Venkataraman and Sreenivasan), 17.

Rice plant, relation between the age, and its susceptibility to helminthosporium and blast diseases (Padmanabhan and Ganguly), 44.

Rooting responses in isolated tropical leaves (Samantarai and Kabi), 243.

Stomach poisons, bioassay, some aspects, studies (Pradhan and Jotwani), 37.

Termite, mound building, Termes (Cyclotermes) *obesus* (Rambur) digestion of cellulose (Misra and Ranganathan), 100.